Rapid Publications

Progression of Nephropathy in Spontaneous Diabetic Rats Is Prevented by OPB-9195, a Novel Inhibitor of Advanced Glycation

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Levels of tissue advanced glycation end products (AGEs) that result from nonenzymatic reactions of glucose and proteins are high in both diabetic and aging people. Irreversible AGE formation is based on increases in AGE-derived protein-to-protein cross-linking and is considered to be a factor contributing to the complications of diabetes. A novel inhibitor of advanced glycation, OPB-9195, belongs to a group of thiazolidine derivatives, known as hypoglycemic drugs; however, they do not lower blood glucose levels. We did studies to determine if OPB-9195 would prevent the progression of nephropathy in spontaneous diabetic rats. In vitro inhibitory effects of OPB-9195 on AGE formation and AGE-derived cross-linking were examined by enzyme-linked immunosorbent assay (ELISA) and SDS-PAGE, respectively. Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats, a model of NIDDM, were used to evaluate the therapeutic effect of OPB-9195. Light microscopic findings by periodic acid-Schiff (PAS) staining, the extent of AGE accumulation detected by immunohistochemical staining in the kidneys, the levels of serum AGEs by AGE-specific ELISA, and urinary albumin excretion were examined. OPB-9195 effectively inhibited both AGE-derived cross-linking and the formation of AGEs, in a dose-dependent manner in vitro. In addition, the administration of OPB-9195 prevented the progression of glomerular sclerosis and AGE deposition in glomeruli. Elevation of circulating AGE levels and urinary albumin excretion were dramatically prevented in rats, even at 56 weeks of age and with persistent hyperglycemia. We concluded that a novel thiazolidine derivative, OPB-9195, prevented the progression of diabetic glomerular sclerosis in OLETF rats by lowering serum levels of AGEs and attenuating AGE deposition in the glomeruli. *Diabetes* 46:895–899, 1997

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AGE, advanced glycation end products; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; OLETF, Otsuka-Long-Evans-Tokushima-Fatty; PAS, periodic acid-Schiff.

Nephropathy is a morbid complication of diabetes. It is characterized by a progressive decline in renal function, and end-stage kidney disease will occur 5 to 10 years later (1). It is widely considered that persistent hyperglycemia is the primary causal factor for development of most diabetic complications, including diabetic nephropathy (2). Mechanisms by which chronic hyperglycemia causes pathophysiological and metabolic changes are essentially unknown.

In recent studies, AGEs were found to play an important role in the pathogenesis of diabetic complications, particularly in the progression of tissue damage leading to eye and kidney diseases (3–6). AGEs, the late products formed from early Amadori products during the Maillard reaction, slowly accumulate in various tissues (4). Direct evidence indicating the importance of AGEs in the progression of diabetic nephropathy has been reported. Administration of exogenous AGEs to normal rats induces glomerular hypertrophy and mesangial sclerosis, gene expression of matrix proteins, and production of various growth factors (7–10). Other studies revealed that aminoguanidine, an inhibitor of AGE formation, ameliorates the structural impairment of glomeruli and prevents proteinuria in STZ-induced diabetic rats (11–13). In addition, the finding of decreased Hb-AGE levels as a result of aminoguanidine therapy in diabetic patients provided direct evidence for the efficacy of AGE inhibitors in humans (14). Effective AGE inhibitors may prevent diabetic complications, and we report here that a novel thiazolidine derivative, OPB-9195 (international application published under the patent cooperation treaty; publication number WO94/19335; publication date 1 September 1994), inhibits the formation of AGEs, even in an extremely small dose, as compared to aminoguanidine, and dramatically prevents the development of diabetic nephropathy in OLETF rats.

RESEARCH DESIGN AND METHODS

OPB-9195 was developed in Otsuka Pharmaceutical, Japan. The chemical structure of OPB-9195, (±)-2-Isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide, is shown in Fig. 1. In vitro inhibitory effects of OPB-9195 on AGE-derived cross-linking and the formation of AGEs were examined. Egg white lysozyme (Sigma Chemical) at a concentration of 100 mg/ml in 200 mmol/l glucose or 100 mmol/l fructose were mixed with various concentrations of OPB-9195 (0–10 mmol/l). All samples were incubated at 37°C. After 7 days of incu-

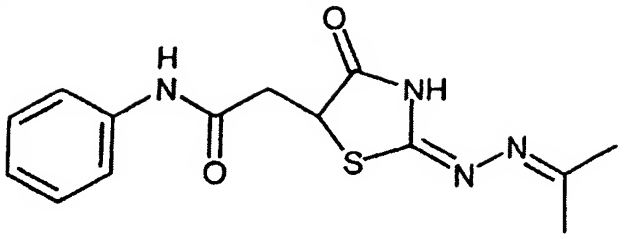

FIG. 1 The chemical structure of OPB-9195, (±)-2-Isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide.

bation, aliquots were removed for determination of AGE-derived cross-linking and levels of AGEs. For evaluation of AGE-derived cross-linking, aliquots were applied to 10–20% SDS-PAGE gels, under reducing conditions, and stained by Coomassie blue. Levels of AGEs in each sample were determined by AGE-specific ELISA, as described previously (16).

To evaluate the therapeutic effects, OPB-9195 was administrated to OLETF rats, a model of NIDDM. OLETF rats were established in the Tokushima Research Institute, Otsuka Pharmaceutical, in 1990 (17) and obtained from Otsuka Pharmaceutical, Japan. Male OLETF rats (weight: 610–650 g) at 24 weeks of age were randomized into a nontreated group (non-Tx; n = 12) and a OPB-9195-treated group (Tx; n = 12). OPB-9195 had been administered to these rats at the concentration of 1 mg/g mixed chow from 24 weeks of age. Four rats in both groups were killed at 24, 44, and 56 weeks of age. Levels of serum AGEs were measured by AGE-specific ELISA, as described previously (16). Urinary albumin and urine creatinine concentration were also measured. For immunohistochemical staining, isolated kidney tissues were embedded in OCT compound and rapidly frozen in –80°C. Frozen sections cut sequentially into 4-μm thickness were incubated with rabbit anti-AGE antiserum (16) in phosphate-buffered saline (PBS) containing 1% of bovine serum albumin (BSA) after blocking nonspecific staining by 10% normal goat serum. Bound antibody was detected by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (Zymed Laboratories). Fluorescence intensity was examined by fluorescence microscopy. Other tissues were fixed in 10% phosphate-buffered formalin, and serial sections of paraffin-embedded tissues were stained with periodic acid-Schiff (PAS), by standard methods. The degree of sclerotic changes was evaluated by the percentage of sclerotic glomeruli and calculating sclerosis index (18) for at least 200 glomeruli in each rat. Differences between the groups were analyzed with unpaired t test. All values are expressed as means ± SD.

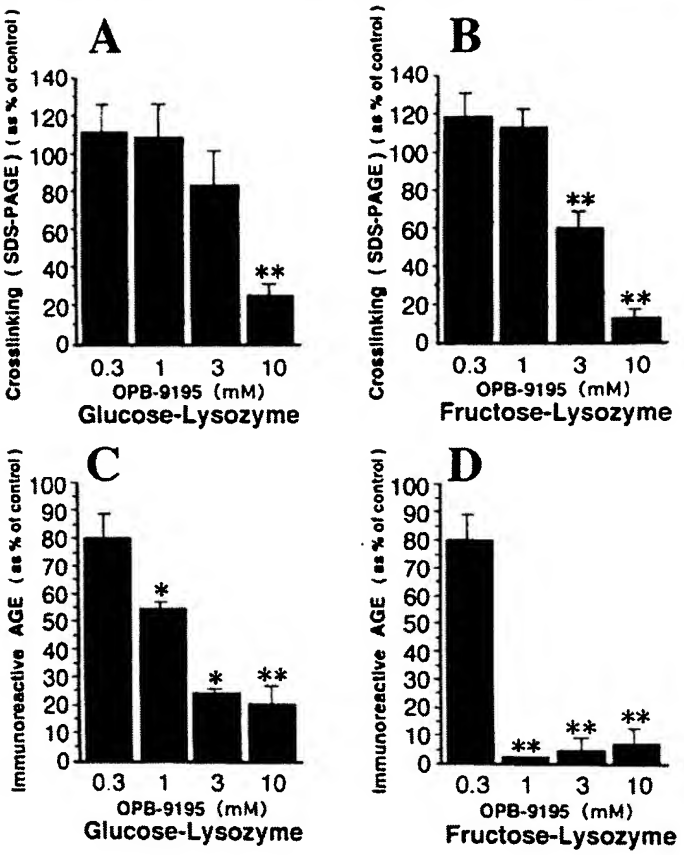

FIG. 2. Effect of OPB-9195 on cross-linking and formation of immunoreactive AGEs in the presence of glucose and fructose in vitro. AGE-derived cross-linking was evaluated by SDS-PAGE (*A*, *B*), and levels of AGEs were determined by AGE-specific ELISA (*C*, *D*). Results are expressed as a percentage of control sample without OPB-9195. Data are representative of three independent experiments. *P < 0.05 vs. 0.3 mmol/l OPB-9195; **P < 0.01 vs. 0.3 mmol/l OPB-9195.

RESULTS

The in vitro inhibitory effect of OPB-9195 for AGE-derived cross-linking and AGE content formed by the incubation of glucose (Fig. 2*A*, *C*) and fructose (Fig. 2*B*, *D*) were examined. OPB-9195 effectively inhibited AGE-derived cross-linking (Fig. 2*A*, *B*) as well as formation of AGEs (Fig. 2*C*, *D*) in a dose-dependent manner.

The increase of plasma glucose levels in the non-Tx group was identified from 24 weeks of age, and serum levels of AGEs increased in parallel with the progression of diabetes. The serum levels of AGEs in the Tx group were significantly lower than in the non-Tx group at 44 weeks and 56 weeks of age (P < 0.01), although no significant difference was seen in plasma glucose levels (Table 1). The percentage of glomeruli that showed focal and segmental glomerular sclerosis at 44 weeks of age was significantly lower in the Tx group than in the non-Tx group (P < 0.05). Sclerosis index was also significantly lower in the Tx group than that in the non-Tx group at 44 and even at 56 weeks of age (P < 0.05, Table 1). Urinary albumin excretion at 44 weeks of age did not differ between

TABLE 1
Effect of OPB-9195 on plasma glucose, serum levels of AGEs, and the degree of glomerular sclerosis in OLETF rats

	Plasma glucose (mg/dl)		AGEs in serum (AGE U/ml)		Focal and segmental glomerular sclerosis (%)		Sclerosis index	
	Administration of OPB-9195							
Age (weeks)	–	+	–	+	–	+	–	+
24	255 ± 37	266 ± 32*	4.9 ± 0.7	4.8 ± 0.5*	0.9 ± 0.9	—	0.30 ± 0.02	—
44	269 ± 43	261 ± 33*	10.0 ± 2.5	5.3 ± 0.6†	9.7 ± 4.1	3.1 ± 1.0‡	0.54 ± 0.06	0.41 ± 0.04‡
56	286 ± 47	266 ± 40*	15.1 ± 2.0	6.9 ± 0.9†	37.6 ± 19.2	18.4 ± 19.9*	0.71 ± 0.01	0.54 ± 0.01‡

Data are means ± SD. Negative (–) and positive (+) symbols show the administration of OPB-9195. *NS; †P < 0.01; ‡P < 0.05 vs. OPB-9195 nontreated group.

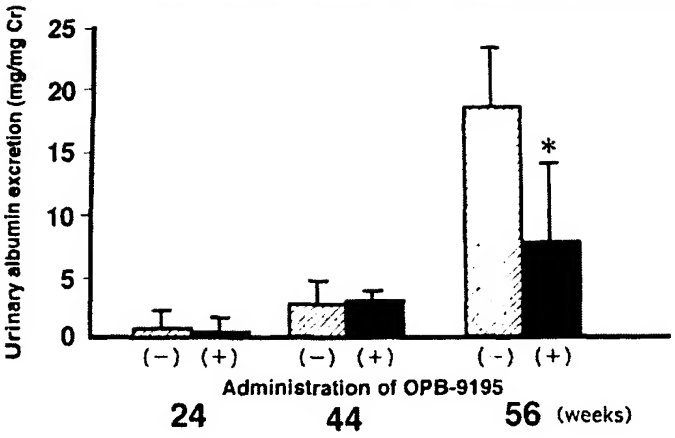

FIG. 3. Effect of OPB-9195 on the urinary albumin excretion in OLETF rats at different ages (four rats per group). *$P < 0.05$ vs. non-treated group.

the Tx and the non-Tx group, but at 56 weeks of age, albumin excretion in the Tx group was significantly low compared with the non-Tx group ($P < 0.05$, Fig. 3).

PAS-positive nodular lesions, thickening of capillary walls, and exudative changes, which are all typical changes in diabetic nephropathy, were present in non-Tx sections in rats at 56 weeks of age (Fig. 4*A*). In contrast, these findings were remarkably diminished in sections of Tx rats (Fig. 4*B*). These histological changes, all suggestive of diabetic nephropathy, were observed in almost 40% of glomeruli in non-Tx rats at 56 weeks of age. Focal interstitial fibrosis and monocyte infiltration were also evident in non-Tx rat sections. In contrast, age-matched rats that had been given OPB-9195 showed only slight proliferation of the mesangial matrix, few sclerotic glomeruli, and less interstitial damage. In addition, distinct AGE deposition in the glomeruli was present in non-Tx rats. Accumulation of AGEs was extensive in the mesangial lesions as well as in the capillary loops (Fig. 4*C*). However, the intensity of AGE deposition in glomeruli of Tx rats was markedly diminished compared to non-Tx rats and was localized within the mesangial lesions (Fig. 4*D*).

DISCUSSION

There is now considerable evidence for major roles of AGEs in the complications seen in diabetic people (4). AGEs may bind mesangial cells and induce production of growth factors and matrix proteins (9,19,20). These observations provide

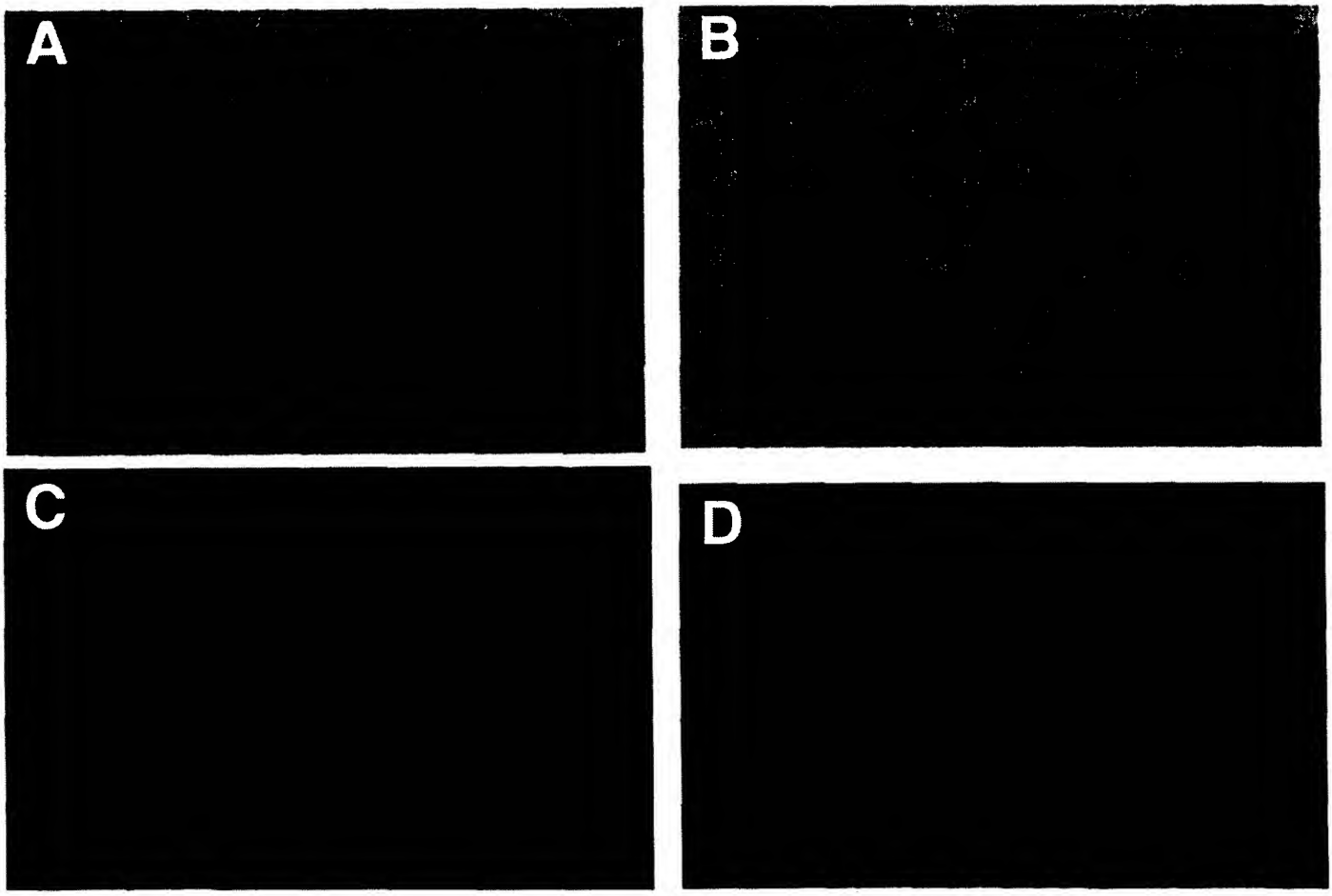

FIG. 4. Light microscopic and immunohistochemical findings in glomeruli of OLETP rats at 56 weeks of age, given or not given OPB-9195. *A*: PAS-stained glomerulus from an OPB-9195 non-treated rat. *B*: PAS-stained glomerulus from an OPB-9195 treated rat. *C*: immunohistochemical staining of glomerulus by antibody against AGEs; OPB-9195 non-treated rat. *D*: immunohistochemical staining of glomerulus by antibody against AGEs; OPB-9195 treated rat.

the rationale for evaluating the efficacy of AGE inhibitors to attenuate the complications of diabetic people.

Aminoguanidine, an inhibitor of AGE formation, has been reported to prevent diabetic microangiopathy, including nephropathy (4,7–13,21). Aminoguanidine at 200 mmol/l inhibited both the formation of fluorescent AGEs and glucose-derived cross-linking in vitro (21). In contrast, inhibitory effects of OPB-9195 at 3 to 10 mmol/l were observed; these are significantly lower doses than that of aminoguanidine used in in vitro experiments (Fig. 2). In in vivo studies on diabetic rats, aminoguanidine was administered in a dose of a total 100 mg/kg i.v. (7), by 50 mg/kg daily intraperitoneal injections (11) or by 1 g/l in drinking water (12). However, even a relatively small dose of OPB-9195 (1 mg/g mixed chow) ameliorated diabetic nephropathy in OLETF rats by inhibiting the accumulation of AGEs in the kidneys (Fig. 4). This inhibition was particularly evident in the capillary loops, compared with the mesangial matrix, and was accompanied by a significant decrease in urinary albumin excretion. Urinary protein excretion is generally considered to correlate with damage to barrier functions in the glomerular basement membrane. Since OPB-9195 significantly decreased albumin excretion at the late period of diabetic nephropathy (Fig. 3), it is possible that the modification of the glomerular basement membrane by AGEs contributes to proteinuria in diabetic nephropathy, as suggested previously (22).

OPB-9195 belongs to a group of thiazolidine derivatives known as hypoglycemic drugs. However, OPB-9195 had no effect on lowering blood glucose levels (Table 1). Although OLETF rats remained hyperglycemic after 24 weeks of age, the oral administration of OPB-9195 clearly prevented the development of diabetic nephropathy, as noted microscopically, and significantly improved sclerosis indexes (Table 1, Fig. 4). Some glomeruli in sections of Tx rats at 56 weeks of age actually showed little sclerotic change; however, the sclerotic area in the peripheral lobules was extremely smaller than that of glomeruli in non-Tx sections, and most of sclerotic lesions in Tx sections were seen at the glomerular hilus. Thus, the capillary loops of the glomeruli in Tx rats practically escaped from sclerotic involvement, resulting in decreased urinary albumin excretion. These findings suggest that even under conditions of persistent hyperglycemia, OPB-9195 can prevent diabetic nephropathy. The potent AGEs inhibitory effects of OPB-9195 may be of clinical importance for the prevention of diabetic nephropathy, since strict glycemic control is not always feasible in all diabetic patients.

We also assessed the levels of AGEs in the blood circulation and the extent of AGE accumulation. OPB-9195 ameliorated both the levels of circulating AGEs and accumulation of AGEs in the kidneys. Circulating AGEs may possibly be an important factor related to the development of renal dysfunction in subjects with diabetic nephropathy (5,6). At this point, we do not precisely know the link between circulating AGEs and AGE accumulation in tissues, although the present study demonstrated that circulating AGEs bind to tissues and show a toxic effect (6,8). Recently, diketone intermediates such as 3-deoxyglucosone (3-DG), a potent protein cross-linking intermediate of the Maillard reaction, showed rapid reactivity in the AGE-modification of proteins (23). In addition, Vasan et al. (24) reported that a prototypic AGE cross-link "breaker," *N*-phenacylthiazolium bromide (PTB), selectively cleaves the diketone bridges in AGE cross-links and suggested that this drug may reverse damage that has occurred. All these findings suggest the therapeutic importance of reducing circulating AGEs and diketone intermediates levels, which cause AGE-derived protein to protein cross-links. In this context, we speculate that OPB-9195 inhibits the accumulation of AGEs in kidney tissues by preventing the bindings of circulating AGEs or diketone intermediates. Newly emerging therapies aimed at inhibiting AGE formation are important to prevent the progression of diabetic nephropathy. Clinical trials to evaluate the efficacy of OPB-9195 as an agent to ameliorate diabetic nephropathy are being planned.

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REVIEW

Diabetes and advanced glycation endproducts

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Abstract. Vlassara H, Palace MR (Department of Geriatrics, Division of Experimental Diabetes and Ageing, Mount Sinai School of Medicine, and Department of Medicine, Division of Endocrinology, Mount Sinai School of Medicine, NY, USA). Diabetes and Advanced Glycation Endproducts. *J Intern Med* 2002; **251**: 87–101.

Bio-reactive advanced glycation endproducts (AGE) alter the structure and function of molecules in biological systems and increase oxidative stress. These adverse effects of both exogenous and endogenously derived AGE have been implicated in the pathogenesis of diabetic complications and changes associated with ageing including atherosclerosis, renal, eye and neurological disease. Specific AGE receptors and nonreceptor mechanisms contribute to these processes but also assist in the removal and degradation of AGE. The final disposal of AGE depends on renal clearance. Promising pharmacologic strategies to prevent AGE formation, reduce AGE toxicity, and/or inactivate AGE are under investigation.

Introduction

Prolonged exposure to hyperglycaemia is now recognized as the primary causal factor in the majority of diabetic complications [1, 2]. Indeed, glucose has a wide range of transient and reversible effects on cell function [3, 4] as well as effects that are irreversible and can cause progressive, cumulative dysfunction [5]. This suggests that persistent, rather than acute, metabolic alterations are of pivotal importance in the development and progression of diabetic complications. Amongst the irreversible changes which occur as a direct result of hyperglycaemia is the formation of advanced glycation endproducts (AGEs) via the Maillard reaction. AGEs have a range of chemical, cellular, and tissue effects and act as mediators, not only of diabetic complications, but also of widespread changes associated with ageing.

The following review focuses on the role that advanced glycation plays in the initiation and progression of diabetic complications. We will also review agents which can ameliorate the toxic effects of these products and speculate about novel therapeutic interventions.

AGE: metabolic aspects

Endogenous AGE

Effects of short-lived AGE molecules. Advanced glycation endproduct formation has been known to have significant effects on macromolecular structure and function. Until recently it was thought that AGE formation involves primarily long-lived extracellular proteins and occurs as a function of time, thus representing a form of molecular senescence. It is now clear that AGEs arise on short-lived molecules

as well, including circulating plasma proteins and lipids, and that their levels are significantly elevated in diabetic patients and in patients with impaired renal clearance [6, 7]. It is also recognized that they can form rapidly on cytoplasmic proteins and nucleic acids. Indeed, it has been demonstrated that intracellular AGEs may form at a rate up to 14-fold faster in high (30 mM) glucose conditions [8]. Such AGE-modification of short-lived molecules is known to involve oxidation of proteins and lipids, to disrupt molecular conformation, to alter enzymatic activity, to reduce degradative capacity, and to result in abnormal recognition and clearance by receptors [9–12].

The broad pathological significance of AGE-modification is best reflected in the glycation of lipids and lipoproteins, as in the case of apoprotein B (ApoB) and low density lipoprotein (LDL) [13]. Dyslipidaemic changes are evident in diabetic patients and are characterized by increased levels of LDL which greatly predispose these patients to atherosclerosis, with subsequent increased risk for coronary heart disease and stroke. Advanced glycation of the lipid component of LDL occurs concomitantly with LDL oxidation *in vitro* [13]. The presence of amino groups on certain phospholipids such as phosphatidylethanolamine and phosphatidylserine provides appropriate sites with which glucose can react with lipid amines to form AGEs [14]. During glycation, fatty acid residues can be oxidized independently of transition metals or exogenous free radical generating systems [14]. Significantly, LDL oxidation follows the formation of AGE–LDL, whilst both occur in direct proportion to glucose concentration and are inhibited in the presence of the AGE inhibitor aminoguanidine [14]. Thus, it is apparent that free amine:glucose interactions with lipids are spontaneous and natural *in vivo* steps leading to fatty acid glycation and oxidation products.

The ApoB component of LDL is a relatively large protein with many potential lysine and arginine AGE modification sites, although the predominant site of such modification has been found distally to the N-terminus of the LDL-receptor binding domain [13]. AGE–ApoB levels are approximately 4-fold higher in diabetic patients [14, 15]. The pathophysiological implications of this AGE-modification have been demonstrated in a study in which AGE–LDL was injected into transgenic mice expressing the human LDL receptor. The clearance of the modified LDL was delayed compared with that of native LDL [15]. This suggests that advanced glycation of ApoB can lead to hyperlipoproteinaemia and, thus, may actively contribute to atherosclerosis by reducing LDL clearance and by facilitating AGE–LDL deposition in the vessel wall via AGE-receptor interactions (see below) [16, 17].

Effects of long-lived AGE molecules. The rate of formation of AGEs exceeds that predicted by first-order kinetics. This implies that, over time, even modest hyperglycaemia can result in significant accumulation of AGEs on long-lived macromolecules [18–20]. This is well illustrated on certain long-lived proteins such as those of the ocular lens. For example, the progressive post-translational modification of lens crystallins by glucose-derived AGEs explains the premature lenticular browning and cumulative crosslinking occurring during the course of diabetes [21, 22]. This accounts for a significant proportion of lenticular opacification and subsequent cataract formation during ageing as well as in diabetes [23, 24]. These changes can be reproduced *ex vivo* in the presence of high glucose concentrations and can be effectively prevented in the presence of the AGE-inhibitor aminoguanidine [25]. Furthermore, the collagen network of human vitreous gel contains increased levels of AGEs in diabetics [25] and vitreous AGE levels show a significant correlation with age, suggesting that AGEs play a major role in diabetes- and age-related vitreous alterations including progression of retinopathy and posterior vitreal detachments (PVDs) [25].

Proteins constituting the extracellular matrix (ECM) and vascular basement membranes (BM), which are amongst the longest lived in the body, are highly susceptible to AGE-modification. Functionally, AGE-mediated crosslinks in BM are known to cause reduced solubility and decreased enzymatic digestion [26, 27]. In addition, AGE formation has been shown to impair the geometrically ordered self-assembly of BM proteins, thereby causing structural and functional abnormalities. For example, AGE-modification of laminin, vitronectin, and collagen can seriously alter molecular charge characteristics, upset the ability to form precisely assembled three dimensional matrix aggregates, and thus disrupt biological attachment sites which enable cells to adhere to their substrates

[11, 28–30]. Moreover, the reduced binding of heparin sulphate proteoglycan matrix to AGE-modified collagen, laminin, and fibronectin significantly alters the polyanionic nature of BM [31, 32], seriously affecting the charge-mediated properties of BM. Thus, the very presence of AGE on vascular BM may have dire pathological consequences, particularly in diabetics, who have an accelerated accumulation of AGEs.

AGE-DNA and embryopathy. Whilst the primary amino groups of nucleotides are less reactive nucleophiles than are ε-amino groups of lysine and arginine, nucleic acids can, nonetheless, react with reducing intracellular sugars to form Amadori and AGE-products with characteristic fluorescence and spectral properties [33]. AGE-formation on DNA can cause single strand breaks in genomic DNA which can have serious teratogenic effects [33–35].

Teratogenic effects may also be promoted in diabetes by enhanced glycation of histone proteins, which have a vital structural role in maintaining nucleosomes and hence DNA integrity. Intracellular sugars such as glucose-6-phosphate and ADP-ribose react strongly with amino groups on histones where they can cause crosslinking *in vitro* [36, 37] as well as *in vivo* [38].

Maternal diabetes has been associated with congenital malformations and increased foetal mortality and morbidity [39]. It is estimated that there is a two to three fold increased incidence of perinatal infant fatalities as a result of congenital malformations in the offspring of women with insulin dependent diabetes compared with those born to nondiabetic mothers [40]. Most studies show a generalized increase in malformations involving multiple organ systems, although the teratogenic mechanisms are largely unknown. From the available evidence it would appear that AGE-mediated DNA damage could be a significant factor in the teratogenicity occurring in diabetic pregnancies. The incidence of congenital abnormalities is not increased in short-term, pregnancy-induced hyperglycaemia, as it is when long-term poor glycaemic control predates conception [41]. This observation, together with the marked reduction in foetal abnormalities with effective management of hyperglycaemia during pregnancy has added further evidence implicating the role of AGEs in diabetic embryopathy.

Major exogenous sources of AGEs

Diet. Recent studies suggest that AGEs introduced to biological systems from exogenous sources such as diet and smoking may have significant impact on disease mechanisms. Diet is the major source of exogenous AGE, with the highest content in complex foods, such as those rich in carbohydrates and fats. Formation of AGE is enhanced by exposure to heat; thus AGE content, which is responsible for the browning of food as it cooks, increases with cooking temperature and duration. Whilst the abundance of dietary AGEs including methylglyoxal (MG) and carboxymethyl lysine (CML) has long been recognized, their significance as potential toxins was not appreciated as their absorption was estimated to be only approximately 10% of that ingested [42].

The availability of new AGE-specific bioassays has facilitated the study of the dietary content, bioavailability and renal elimination of these moieties, schematically depicted in Fig. 1. Koschinsky et al. [43], showed that AGE immunoreactivity can increase by 200-fold in egg- whites cooked with fructose, compared with egg-whites prepared in the identical manner in the absence of fructose. This study also confirmed the absorption of 10% of ingested AGE and showed that only one-third of that absorbed is excreted within 48 h in the urine of patients with normal renal function [43]. As AGEs that are not cleared by the kidney are distributed to the tissues where they remain biologically active [43], the data imply that dietary AGEs may pose a significant environmental risk, particularly to patients with nephropathy.

The contribution of dietary AGEs to injury *in vivo* is apparent from a study in which mice were randomized to either a high or low AGE diet, both isocaloric and identical in protein and nutrient content; mice on the high AGE diet exhibited significant albuminuria as well as microscopic evidence of glomerular hypertrophy and/or sclerosis, effects not seen in the mice fed the low AGE diet (Fig. 2) [44].

Other *in vivo* evidence of the role of dietary glycotoxins was obtained from a study which maintained ApoE-deficient mice on chow which was either high or low in AGE content for a total of 5 weeks. After 1 week, a femoral artery denudation injury was induced and the designated diet was maintained for another 4 weeks before the injured arteries were

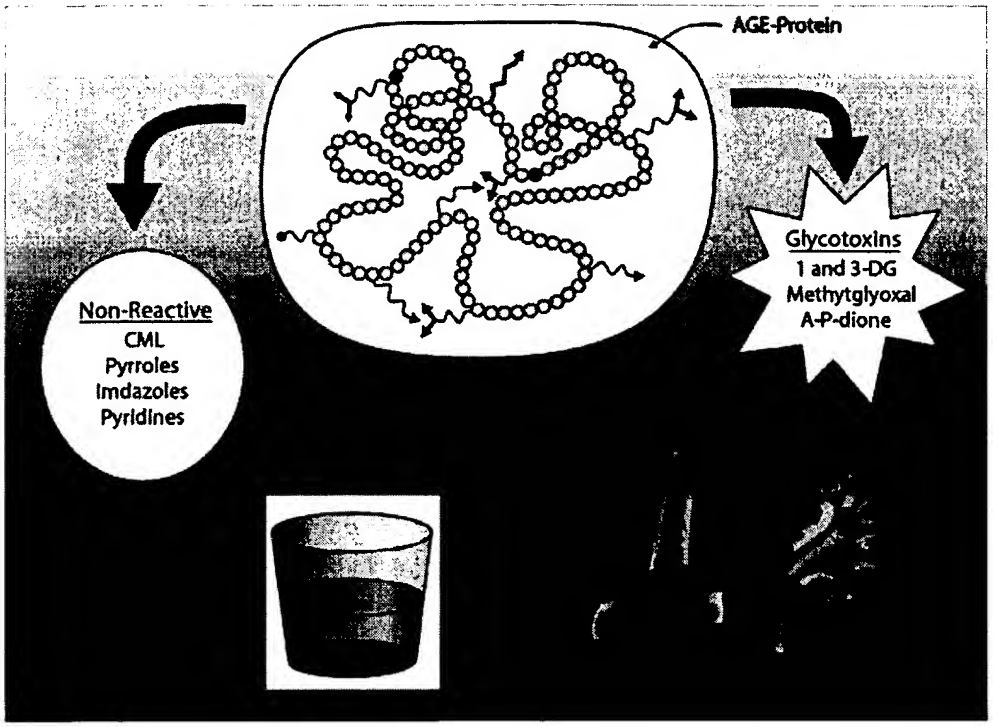

Fig. 1 Schematic representation of the fate of diet-derived AGEs. Cooked foods contain sugar-derived protein or lipid glycation intermediates that may include noncrosslinking products, such as *N*-ε-carboxymethyl-lysine (CML), pyrroles, imidazoles, pyridines (left insert), or crosslink forming, reactive intermediates (glycotoxins), such as 1-,3-deoxyglycozone (3-DG), methylglyoxal, protein-linked A-P-dione (right insert). The former are presumably readily excreted in urine, whilst the latter may reattach onto serum or tissue components to form new AGEs with the eventual pathological consequences.

examined. Injured arteries in the animals fed with the low-AGE diet showed a significant reduction in neointimal area (Fig. 3). In addition, those animals developed less complex lesions with fewer foam cells within the neointima, associated with a 40% decrease in serum AGE levels [45].

Similarly, Apo-E deficient streptozotocin diabetic mice which were fed with a diet with AGE content 10-fold lower than that of regular rodent chow had significantly suppressed aortic atherogenesis compared with animals on the regular chow. This was associated with significantly reduced serum AGE levels despite elevated lipid levels [46].

A recent study in humans compared the effects of two diets; both were compatible with American Heart Association and American Diabetes Association guidelines but they differed 6-fold in their AGE content. Eleven diabetic subjects with normal renal function were enrolled in the crossover study; they consumed each diet for a 2-week period separated by a 2-week washout. After 2 weeks on the low AGE diet, there was a significant decrease in fasting serum AGE levels. Furthermore, blood mononuclear cell expression of TNF-α mRNA and serum levels of vascular cell adhesion molecule-1 (VCAM-1), which were significantly higher at the end of the high AGE diet period, decreased by approximately 30–50% at the end of the low AGE diet period [47]. The fact that reduction in dietary AGE content resulted in lower levels of inflammatory mediators in humans, as well as the animal data above [45, 46], underscore the potential role of a low AGE diet in the primary prevention of atherosclerosis as well as in the prevention of restenosis after coronary angioplasty.

Smoking. As tobacco leaves are dried in the presence of sugars, a process called curing, the Maillard reaction cascade leads to the formation of glycated and oxidized derivatives. Upon combustion, reactive AGE species are volatilized. These species, also termed 'glycotoxins', are inhaled, absorbed through the lungs and become conjugated with serum proteins,

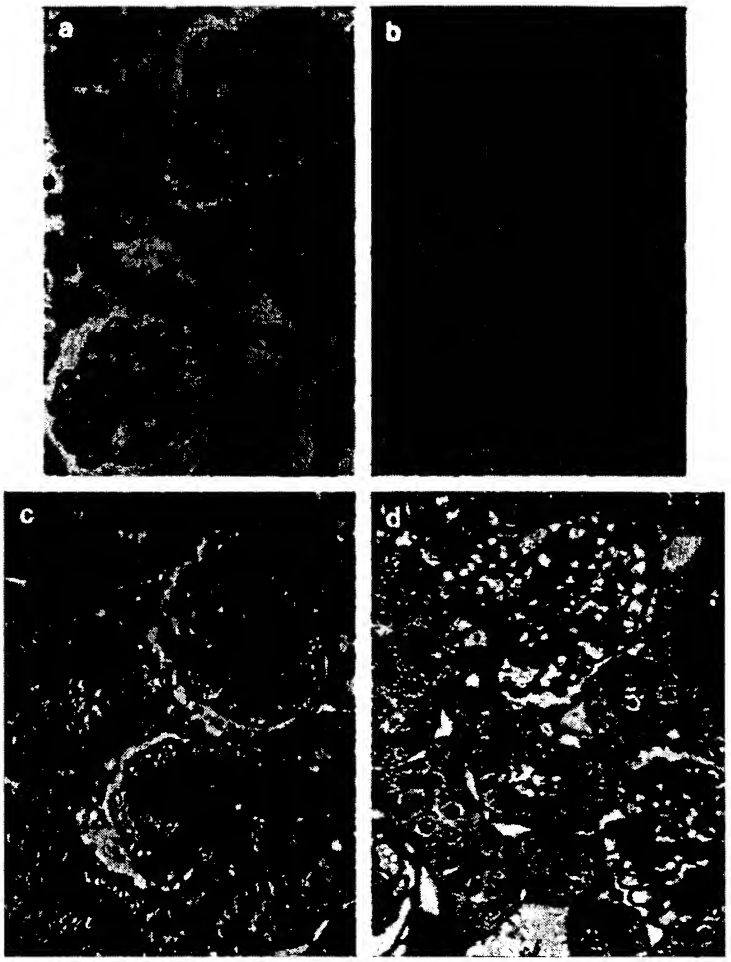

Fig. 2 Glomerular disease development is delayed in kidneys from diabetic NOD and db/db mice fed a regular diet of low AGE content. Panels a, b: db-NOD mice after 2-months on diet (H&E); Panels c, d: db/db mice after 5 months (H&E); Panels a, c: Standard mouse chow (high in AGE); Panels b, d: Same diet containing 5-fold lower AGE.

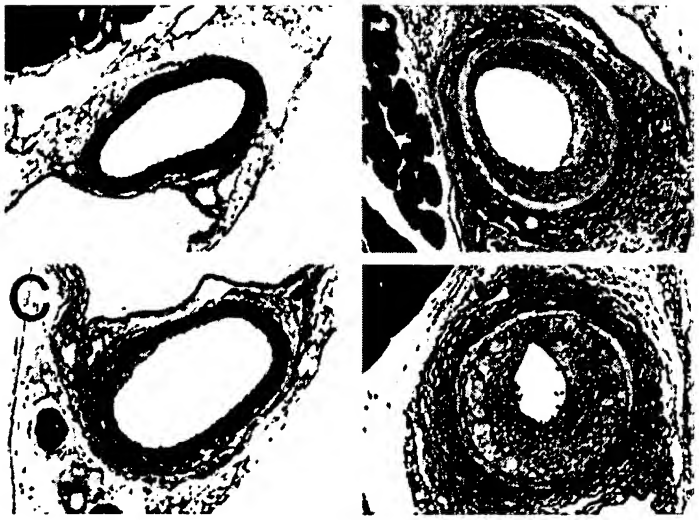

Fig. 3 Postinjury arterial restenosis is delayed in ApoE-deficient mice fed low AGE diet. Cross sections of normal mouse femoral arteries 4 weeks after injury. A and C: sham operated, uninjured controls; B and D: injured arteries. ApoE mice were fed with either low AGE diet or high AGE diet for a total of 5 weeks. Injured arteries underwent transluminal endothelial denudation within 1 week after diet (CME staining; magnification × 200).

including lipoproteins [48]. This is reflected in the fact that total serum AGE and AGE-apoprotein B levels in cigarette smokers were found to be significantly higher than in nonsmokers [48]. Also, smokers, and especially diabetic smokers, had high AGE levels in their arteries [49, 50] and ocular lens [50].

AGE: cellular interactions

Specific and nonspecific AGE-receptor systems

Early in AGE research it was speculated that a natural receptor-based system existed *in vivo* whereby AGE products could be removed from tissues, thereby limiting their deleterious effects. Work conducted over the last 15 years has led to the recognition of a complex AGE receptor system which appears to play a key role in AGE-related biology as well as in the pathology associated with the complications of diabetes and ageing [51]. It was originally demonstrated that AGE-modified proteins are recognized by specific receptors which are unrelated to previously described scavenger receptor systems [52]. In recent years, several AGE-binding molecules have been described.

Two AGE-binding proteins, a ~60 kDa (p60) and ~90 kDa (p90), isolated from mouse macrophages and rat liver membranes, were initially identified [53, 54]. P60, now referred to as AGE-R1, with characteristic membrane-spanning and signal domains, was shown to be homologueous to a ~50 kDa component of the oligosaccharyltransferase complex (OST 48) [55], whilst the p90, also termed AGE-R2, with complete homologuey to an 80-kDa protein, was found to be a phospho-tyrosine containing protein [56] linked to early signalling via several adaptor molecules, e.g. Shc and Grb2. An additional 32 kDa AGE-binding protein with links to the AGE receptor complex (AGE-RC) was described shortly afterwards and is known as AGE-R3. This protein, also called Galectin-3, Mac-2, or carbohydrate binding protein-35, exhibits high-affinity binding for AGE ligands [57].

Evidence indicates that AGE-R1 and -R3 are largely responsible for AGE-recognition and high-affinity binding. AGE-R2 was shown to be subject to AGE-induced phosphorylation [58]. The R2 component of the AGE-R may thus play a role in signal transduction and cell activation associated with AGE receptor binding [59, 60].

Amongst the other cell-associated receptors interacting with AGEs are RAGE [61, 62], ScR-II [63, 64] and CD36 [65]. RAGE, a multiligand member of the immunoglobulin superfamily, though not as efficient in AGE endocytosis and turnover [66], is viewed increasingly as an intracellular signal-transducing, or pro-inflammatory, peptide. In this regard, RAGE may be more accurately classified in the family of oxidant-stress inducing signalling molecules or cofactors. Consistent with the above, in animal models, brief infusion of soluble truncated RAGE is reported to intercept diverse processes such as endothelial leakage, atherosclerosis, and inflammatory bowel disease [61, 66]. In addition, specific and saturable binding of AGE-BSA to CD36-CHO cells supports the role of CD-36 as a receptor for AGE-proteins [65]. As with RAGE, this scavenger receptor system which is highly expressed on macrophages, although not restricted to AGE uptake, may contribute to AGE-mediated cellular changes, particularly in the context of atherosclerosis and foam cell formation [63, 67, 68].

A more recently discovered molecule with significant AGE-binding affinity and intriguing anti-AGE properties is the known host-defense protein Lysozyme (LZ). Lysozyme is a well-characterized, naturally occurring antimicrobial protein that exerts its effect through the catalytic degradation of the peptidoglycan component of the bacterial cell wall [69]. The AGE-binding site was mapped to a 17-amino acid-long hydrophilic domain, bound on both sides by cysteines, located within one of the two LZ catalytic regions. This AGE-binding cysteine-bounded domain is termed ABCD loop [70]. The previously unrecognized high affinity of lysozyme for AGEs (Kd = 50 nM) included tissue-reactive derivatives found in circulation, suggesting that LZ could be used for the capture and disposal of toxic AGEs formed *in vivo* [70, 71].

Recent reports have shown that LZ administration to nonobese diabetic (NOD) and db/db (+/+) mice normalized serum levels of AGE (sAGE), increased urinary AGE clearance, and improved albuminuria in both diabetic animal models [72, 73]. In additon, in cultured mesangial cells, LZ could suppress the AGE-enhanced expression of several important modulators of kidney structure and function such as PDGF-B, α1 type IV collagen, and tenascin mRNA. LZ, which appears to be expressed by mesangial cells, also normalized the AGE-suppressed MMP-9 gene expression and activity [74]. Thus, LZ revealed several novel anti-AGE properties including enhanced AGE-turnover by macrophages, suppression of AGE pro-inflammatory events, and improved renal AGE clearance. The mechanisms involved in these findings are under investigation.

AGE-receptor regulation

Different activities of the AGE-receptor system have been reported to be modulated by diabetic factors, e.g. glucose, insulin, AGEs, and reactive oxygen species (ROS) in many types of cells [75–78]. Most commonly used measures for assessing AGE-receptor modulation have centred on parameters of cell activation.

Advanced glycation endproduct-stimulated inflammatory cytokine production by macrophages and its autoregulatory impact on receptor expression and endocytic activity were amongst the original observations which broadened the concept of a system known mostly for its extracellular physico-chemical properties. Since the first findings, much more intricate bioactive mechanisms have emerged which have linked together sensors of intracellular oxidant stress and inflammatory responses to structural/mechanical tissue functions, all of which have implications in early development and growth, as well as in diverse chronic conditions throughout the body.

The link between AGE-receptor up-regulation and cellular activation has been confirmed for a number of the presently identified receptor components. However, cell activation can occur by nonreceptor pathways, or by intracellularly generated glycoxidant derivatives leading to ROS generation and oxidant stress [53, 79]. Cell activation is also induced in systemic diseases such as hyperlipidaemia, uraemia, amyloidosis, and Alzheimer's disease, all of which are associated with elevated AGE, amongst other metabolites. This makes the receptor's role much more difficult to define.

Much less is known about the regulation of receptor-dependent AGE endocytosis and catabolism. This may be attributable largely to the pleiomorphic and often unstable nature of AGE-ligands. A limited number of studies have suggested either enhancement or suppression of receptor-dependent AGE-degradation in cells from diabetic or ageing animals and humans [67, 76–78].

The macrophage AGE-receptor system, which is the one most closely linked to AGE-turnover, was initially thought to include autoregulatory switches, allowing it to respond to rising AGE levels and to reduce tissue damage [76, 77]. However, the means by which this balance would be maintained *in vivo* during times of excessive AGE accumulation remains largely unknown. As yet undefined metabolic or genetic factors could tip this balance towards pro-inflammatory events via receptor components tied to signalling, e.g. RAGE, AGE-R2, or AGE-R3, or else by receptor-independent mechanisms [53–55, 61, 67]. Alternatively, it could be that delayed AGE processing and disposal, as a result of the lack or malfunction of the endocytotic portion of the AGE-receptor, could promote cytotoxic inflammatory events, thus tipping the balance in the direction of organ damage as shown experimentally in AGE-R3 deficient mice [80, 81].

The first indication supporting this hypothesis in nature was based on findings of suppressed AGE-receptor uptake and degradation, combined with high circulating and kidney tissue AGE levels in NOD mice [82, 83]. These findings were the first to raise the importance of AGE-R1 as a site specific for cell-mediated AGE-processing and degradation. These data were also the first to suggest an *in vivo* functional impairment of receptor-dependent AGE turnover.

In support of these animal data, reduced expression of AGE-R1 in human PBM and in immortalized lymphoblasts from type 1 diabetic (T1D) patients with severe diabetic nephropathy was associated with elevated serum AGE levels and severe diabetic complications [84, 85]. These data indicate that genetic modulation of this receptor system, via means that are not yet recognized, may contribute to organ damage in complication-prone patients.

Genetic analysis of AGE-receptors

For a number of the AGE-receptor-related molecules, the genomic organization and chromosomal location [61, 86–88], as well as several prevalent gene polymorphisms [89, 90], have come to light. Recently, screening for mutations was performed in 48 T1D patients with or without nephropathy, using single strand conformational polymorphism analysis (SSCP) and direct sequencing of allelic PCR fragments [89]. Thus far, none of the polymorphisms found exhibited a clear connection to diabetic complications. However, further investigations are in progress to establish the significance of gene polymorphisms in unexplored regions of AGE-R1 and other molecules, in order to determine whether a primary or secondary genetic link exists.

Degradative mechanisms for removal of AGE-modified molecules

Although endogenous AGE formation can be moderated by lowering blood glucose levels and with the help of various natural antioxidant systems, AGEs eventually accumulate on long-lived macromolecules. Removal of existing AGE-crosslinks from tissue components is conducted largely through extracellular proteolysis and by scavenger cells such as tissue macrophages which ingest AGEs via AGE-specific or nonspecific, e.g. scavenger, receptors. It is also becoming clear that mesenchymal cells such as vascular endothelium or mesangium may also play an important role in AGE-removal. The need for efficient AGE elimination may account, in part, for the increased endocytic activity of vascular endothelium under conditions of high glucose *in vivo* and *in vitro* [91, 92]. Generally, AGE-modified molecules are recognized and internalized by cell-surface receptor-mediated endocytosis, degraded intracellularly, and subsequently released as low molecular weight AGEs, known as 'second generation AGEs' [93]. Whilst these second generation AGEs include reactive intermediates with high crosslinking or oxidative reactivity, their effects may be limited by renal excretion [6, 7, 94–96]. Thus, the overall efficiency of the AGE removal system depends on renal clearance. Kidney dysfunction, as occurs in patients with nephropathy, results in failure to clear circulating AGEs and accounts, in large part, for the marked elevation of serum and tissue AGE levels observed in such patients [6, 7, 94–96]. This mechanism may also contribute to the acceleration of extrarenal vascular damage in patients with end stage renal disease (ESRD).

It is also becoming evident that intracellular protective systems exist to limit the accumulation of reactive AGE intermediates. One such system involves the degradative glyoxalase enzymes which can metabolize the reactive dicarbonyl MG to *S*-d-lactoylglutathione, employing reduced glutathione as a cofactor. The utility and efficiency of such systems is supported in studies in which glyoxalase-1 expression was up-regulated by gene transfection in endothelial cells, resulting in significant inhibition of AGE-mediated cell abnormalities such as increased endocytosis [97].

AGEs as mediators of diabetic vascular complications

Macroangiopathy

Diabetics are more likely to develop serious cardiovascular and cerebrovascular disease than are nondiabetics and are at increased risk for stroke and myocardial infarction caused by vascular occlusion [98–100]. Plaque formation is the most common pathophysiological hallmark of the occlusive process. The interactions between blood borne components, cytokines, growth factors, and the different vessel wall cell types which contribute to atherogenesis is extremely complex and multifactorial [101]. Atheromatous plaque formation in diabetics is indistinguishable from that occurring in nondiabetics, although the distribution of plaques may be different and diabetic lesions characteristically show a higher tendency for focal medial calcification [100]. The occurrence of fatty streaks secondary to the accumulation of lipids and lipoproteins in the vessel wall is an important early step in the evolution of advanced atherosclerotic lesions. It has been widely speculated that oxidative modification of LDL (ox-LDL) *in vivo* results in its reduced recognition by the normal LDL receptor [102, 103], leading to delayed clearance, increases in serum LDL levels [104, 105], and eventually enhanced uptake of the ox-LDL by scavenger receptors on macrophages and vascular smooth muscle cells. As mentioned earlier, advanced glycation of LDL is a physiologically relevant modification which can occur concomitantly with oxidation of LDL *in vitro* [14]. Indeed, recently, AGEs have been accepted as having an important role in the formation and acceleration of atherosclerotic lesions even in normoglycemic patients [17], but especially in diabetics [106, 107] and more so in diabetics with renal insufficiency [108].

Advanced glycation endproducts have been detected within atherosclerotic lesions in both extra- and intra-cellular locations [17, 108–111]. We have recently reported a significant correlation between serum AGE–ApoB and AGE levels in the vessel wall of carotid arteries from nondiabetic patients with occlusive disease requiring endarterectomy [17]. A similar correlation was also demonstrated between

serum AGE–LDL and AGE-immunoreactivity in vascular tissue sections from these patients [17]. Significantly, enhanced AGE-R1 and AGE-R2 were identified in cellular components of atherosclerotic lesions, with a distribution pattern consistent with enhanced tissue AGE immunoreactivity, in the same series [16, 17].

Vascular endothelium expresses receptors for AGEs [55, 58, 61, 62] and it is likely that AGE–LDL is endocytosed by the endothelium via these receptors, leading directly to the accumulation of AGEs in the subendothelial space. In addition, it has been speculated that intracellular accumulation of AGEs may promote phenotypic conversion of smooth muscle cells and foam cell formation within the atherosclerotic plaque. This is consistent with the diffuse pattern of AGE deposition and endocytosis by endothelium, smooth muscle cells and macrophages [106–112]. It also suggests that the process begins early, persists during the entire atheroma formation, and is accelerated during diabetes.

It is now understood that AGE adducts residing in the vessel wall can interfere with endothelium derived nitric oxide synthase and the vasodilatory action of nitric oxide (NO.) [113, 114]. AGEs react directly to inactivate NO-mediated vasodilatation [114] whilst AGE-infused nondiabetic animals show diabetic-like disruption of vaso-relaxation [115]. These alterations may contribute to hypertension, renal impairment and male impotence seen in diabetic patients. Endothelial dysfunction leading to enhanced procoagulant activity may result from exposure of endothelial cells to AGEs *in vitro* [116]. Furthermore, vascular endothelial cells may react to AGEs by promotion of cell adhesion and transendothelial migration [60, 117]. Thus, by causing significant dysfunctional changes in the macrovascular endothelium, AGEs can potentiate vessel wall atherogenesis, hypertension, or prothrombiotic events independent of, but enhanced by, diabetes.

Microangiopathy

Diabetic microangiopathy is a broad term that describes dysfunctional changes in microvascular beds in which endothelium and associated mural cells are progressively damaged, resulting in capillary occlusion, ischaemia, and organ failure. Whilst these abnormalities are most obviously manifested in the kidneys and retina of diabetics, microangiopathy can occur in a wide range of tissues. In fact, damage to the microvasculature in peripheral nerves is now becoming recognized as a major pathogenic factor in diabetic neuropathy [118, 119]. Recently, AGEs have been increasingly implicated in the pathogenesis of diabetic microangiopathy [120, 121]. However, their role in diabetic nephropathy and retinopathy is still under intense investigation.

Diabetic nephropathy is characterized by increased glomerular BM thickening [79] and mesangial ECM deposition, followed by mesangial hypertrophy and diffuse and nodular glomerulosclerosis [122]. Loss of glomerular function is accompanied by a reduction in filtration capacity, culminating in complete renal failure [123]. Structural changes in the glomerulus during diabetes are accompanied by several biochemical abnormalities including the accumulation of AGEs. Immunohistochemical studies of kidney from normal and diabetic rats have suggested that glomerular BM, mesangium, podocytes, and renal tubular cells accumulate high levels of AGEs. Ultrastructural studies have demonstrated BSA–AGE gold conjugate binding to glomerular structures of rats [124] and have indicated that AGE peptides may be reabsorbed by the renal proximal tubular cells [125]. AGE deposition can lead to glomerulosclerosis and widespread dysfunction independent of diabetes [126–128]. This had already been suggested by studies in which normal, nondiabetic animals were administered AGE species-specific albumin. Chronic infusion of nondiabetic animals with AGE-albumin resulted in glomerular hypertrophy, BM thickening, mesangial ECM expansion, and albuminuria, all consistent with a glomerular pathology resembling diabetic nephropathy [51, 115].

The prevalence of diabetic retinopathy in type 1 diabetic patients with T1D for more than 10 years is around 80% and diabetic retinopathy remains amongst the leading causes of blindness in the USA [129, 130]. Diabetic retinopathy is principally a disease of the intraretinal blood vessels, which become dysfunctional in response to hyperglycaemia, with progressive loss of retinal pericytes and eventually of endothelial cells, leading to capillary closure and widespread retinal ischaemia. As in other vascular beds, AGEs have been localized in the retinal vessels of diabetics [75, 131]. The precise role that

these adducts play in the pathogenesis of diabetic retinopathy remains ill-defined, although experimental studies have demonstrated that AGEs may be responsible for some retinal pathology [131] and that AGE-inhibitors, such as anti-glycated albumin [132] or aminoguanidine, can prevent the development of diabetes-associated retinal vascular lesions in rats [133] and in dogs [134]. Interestingly, aminoguanidine does not prevent the initial phase of experimental diabetic retinopathy in rats [135], although a secondary intervention study with this drug has been shown to retard disease progression [136].

Anti-AGE strategies

The determination of the mechanisms of AGE toxicity has been a key strategy in the attempt to prevent diabetic complications. To date there have been several approaches which seek to prevent AGE-formation, reduce AGE effects on cells and break pre-existing AGE crosslinks.

Amadori product formation is the basis of advanced glycation biochemistry because progression to protein crosslinks requires slow rearrangement of the Amadori to create reactive intermediates that can react with amino groups prior to the formation of irreversible AGEs. An important pharmacological strategy for the inhibition of this process has utilized the small nucleophilic hydrazine compound aminoguanidine, a potent inhibitor of AGE-mediated crosslinking [137]. The terminal amino group of aminoguanidine, by virtue of its low pKa, reacts specifically with glucose-derived reactive intermediates to prevent crosslinking. Aminoguanidine has been shown to prevent diabetes related vascular complications in experimental animals by numerous workers [14, 133, 134, 138–145]. From these extensive studies, it is apparent that aminoguanidine could be used to prevent AGE mediated tissue damage caused by diabetes and ageing. In humans, a phase I clinical trial of aminoguanidine measured advanced glycation modified haemoglobin (AGE-Hb) in treated and untreated diabetic subjects and found that AGE-Hb, as well as LDL, was significantly reduced in the treated group. HbA1c values were not affected by aminoguanidine treatment, pointing to the specificity of aminoguanidine for inhibition of post-Amadori, advanced glycation reactions. Aminoguanidine and related AGE inhibitors may eventually find widespread use in diabetics or in individuals at risk for age related vascular sequelae. Other AGE-inhibiting drugs have been under development including the thiazolidine derivative OPB-9195 which has been shown to prevent the progression of diabetic glomerulosclerosis in rats [146].

Prevention of the interactions between AGEs and their receptors or other body proteins is a valid therapeutic approach. The use of neutralizing antibodies against glycated albumin has been shown to prevent BM thickening in diabetic db/db mice without altering the glycaemic status of the animals [132]. Likewise, the AGE-binding properties of lysozyme [70] have been used to reduce AGE levels in the dialysate from diabetic patients with kidney disease [147]. The protocol involves the capture of *in vivo*-derived AGEs with lysozyme linked to a Sepharose matrix allowing the selective depletion of AGEs from sera or dialysate [147]. This approach offers the potential for more efficient reduction of toxic AGEs in body fluids of patients with renal failure by dialysis as well as by *in vivo* lysozyme administration [72, 73].

Recently, a promising therapeutic strategy has been to attack the irreversible intermolecular AGE crosslinks formed in biological systems. This is indeed an exciting approach because it aims to 'break' preaccumulated AGEs and subsequently clear them via the kidney [148, 149]. Such an AGE crosslink 'breaker' prototype, N-phenyl-thiazolium bromide (PTB) has been described to attack covalent carbon-carbon bonds of dicarbonyl-derived crosslinks *in vitro* [148]. More recently, such an AGE-breaker named ALT-711 was found to be capable of reversing AGE-mediated vascular stiffness and distensibility in diabetic rats [150]. If equally effective in the humans, such agents may prove highly beneficial in the reversal of late cardiovascular complications that afflict older adult populations, regardless of diabetes.

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Hydrazine-Conjugated Cellulose for Adsorption of Glycated Proteins

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Abstract: Glycated proteins and advanced glycation endproducts are postulated to play an important role in the development of complications due to diabetes mellitus or renal failure. The purpose of this study was to assess the ability of hydrazine-conjugated cellulose to adsorb glycated albumin. The samples used for adsorption test were artificially glycated albumin or the serum from patients with diabetes mellitus. The hydrazine-conjugated cellulose adsorbed more than 20% of the albumin glycated artificially over 77 days. The serum concentrations of glycated albumin in patients with diabetes mellitus (39.6 ± 2.5% (mean ± SEM), n = 14) were not decreased by incubation with cellulose alone (postincubation level, 39.7 ± 2.5%) whereas they were significantly ($p < 0.0001$) reduced to 38.0 ± 2.4% after incubation with hydrazine-conjugated cellulose. There was a significant correlation ($r = 0.75$, $p < 0.01$) between the preincubation levels of glycated albumin and the degree of adsorption. The hydrazine-conjugated cellulose has a higher affinity for albumin with greater glycation. **Key Words:** Glycated albumin—Glycosylation—Diabetes mellitus—Hydrazine—Advanced glycosylation endproducts.

In our country, hemodialysis treatment is newly instituted every year in about 10,000 patients due to diabetes mellitus. Numerous studies have shown that histological abnormalities are generally observed in the glomerulus and vascular walls about 10 years after the onset of diabetes mellitus. These abnormalities seem to be ascribed to hyperglycemia. Proteins exposed to aldose sugars undergo nonenzymatic glycation and oxidation. The initial, reversibly formed products of this interaction are the Schiff bases/Amadori products, which further undergo irreversible molecular rearrangement, resulting in the formation of so-called advanced glycation endproducts (AGE). AGE are thought to be a cause of diabetic nephropathy. Immunohistochemically, AGE are detected in an expanded mesangial matrix, especially in nodular lesions (1). Circulating AGE peptides have been reported to be increased in patients with end-stage renal failure, in particular those with diabetic nephropathy (2). Current renal replacement therapies including hemodialysis and continuous ambulatory peritoneal dialysis (CAPD) have been insufficient for removing AGE that bind rapidly with proteins (3).

The carbonyl group in ketoamines of glycated protein has a high reactivity with the amino group. Aminoguanidine, which has such an amino group, is now under clinical investigation for the prevention of complications due to diabetes mellitus (4). Ogino and Tani have devised an adsorbent in which the immobilized amino group was used as ligand of glycated proteins (5). The purpose of the current study was to further characterize the adsorbent and to evaluate the grade of adsorption of glycated albumin in the serum samples from the diabetic patients.

METHODS

Synthesis of adsorbent for glycated proteins

Cellulose, which has an exclusion limit against the spherical proteins with molecular weights larger than 400,000, was used as the carrier of the adsorbent (5). Cellulose was incubated with epichlorohydrin, a solvent of cellulose, in a sodium hydroxide solution at 40°C for 2 h. Cellulose was thus altered to epoxy activated gel, which was washed 2 times with an adequate quantity of water and then incubated with a

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surplus amount of hydrazine ($NHNH_2$). The purpose of the surplus amount was to increase the number of hydrazine radicals as much as possible in the epoxy activated cellulose gel. The unbound hydrazine was discarded by washing the gel with a phosphate buffered saline (PBS) solution. Hydrazine, thus conjugated with cellulose, was used as an adsorbent of glycated proteins.

Preparation of glycated human albumin

Human serum albumin (50 mg/ml) and glucose (250 m*M*) in the PBS solution were incubated at 37°C for different periods of 8, 14, 77, or 110 days. The solution was dialyzed against the PBS solution to remove glucose.

Adsorption of artificially glycated albumin

The hydrazine-conjugated cellulose and the cellulose alone were each mixed with 0.9% saline so that the final packed volume of these compounds was 50% of the whole solution. One milliliter of solution of hydrazine-conjugated cellulose or cellulose alone was mixed with 2 ml solution of artificially glycated albumin, and the mixture was incubated at 37°C for 2 h. The incubation was done in association with agitation of the incubator. After centrifugation at 2,000 rpm for 5 min, the concentration of glycated albumin in the supernatant was estimated by the fructosamine concentration. The fructosamine concentration was measured using a commercial kit (6). The efficiency of adsorption (adsorption rate) was calculated by the following formula:

$$[(\text{cellu}) - (\text{cellu-NHNH}_2)] \times 100/(\text{cellu}) \quad (1)$$

In the above formula, (cellu) and (cellu-$NHNH_2$) are fructosamine concentration in the supernatant after incubation with cellulose alone and hydrazine-conjugated cellulose, respectively.

Adsorption of glycated albumin in the serum samples from diabetic patients

Serum samples were taken from 14 diabetic patients with their fasting plasma glucose levels over 200 mg/dl. One milliliter of serum sample was incubated with 1 ml solution of hydrazine-conjugated cellulose or cellulose alone at 37°C for 2 h. After centrifugation at 2,000 rpm for 5 min, the concentration of glycated albumin in the supernatant was estimated by a high performance liquid chromatographic (HPLC) method (7). The difference between the concentration before and after treatment with adsorbent was regarded as the adsorbed portion of glycated albumin. The concentrations of glycated albumin were expressed in percentages of total albumin.

All data are presented as means ± SEM. Comparisons between 2 groups were undertaken using a paired *t*-test. Pearson's coefficient of correlation was used to analyze the relationship between 2 variables. Values of p less than 0.05 were considered significant.

RESULTS

The fructosamine concentration increased in proportion with the duration of glycation. Figure 1 shows the relationship between the duration of glycation and the degree of fructosamine adsorption. The adsorption of fructosamine by the hydrazine-conjugated cellulose increased with the duration of glycation, indicating that albumin with a greater grade of glycation had a higher affinity for the adsorbent. Hydrazine-conjugated cellulose adsorbed more than 20% of the glycated albumin that was produced by glycation over 77 days.

The mean serum concentration of glycated albumin in the diabetic patients was 39.6 ± 2.5% and ranged between 23.5% and 52.8%. The value was not altered by incubation with the cellulose alone and was 39.7 ± 2.5% after incubation. As a result of the incubation with hydrazine-conjugated cellulose, the concentrations of glycated albumin were reduced in all samples. The mean concentration after the incubation was 38.0 ± 2.4%, significantly ($p < 0.0001$) lower than that with cellulose alone (Fig. 2). There was a significant correlation ($r = 0.75$, $p < 0.01$) between the concentration of serum glycated albumin and the adsorbed amount (Fig. 3).

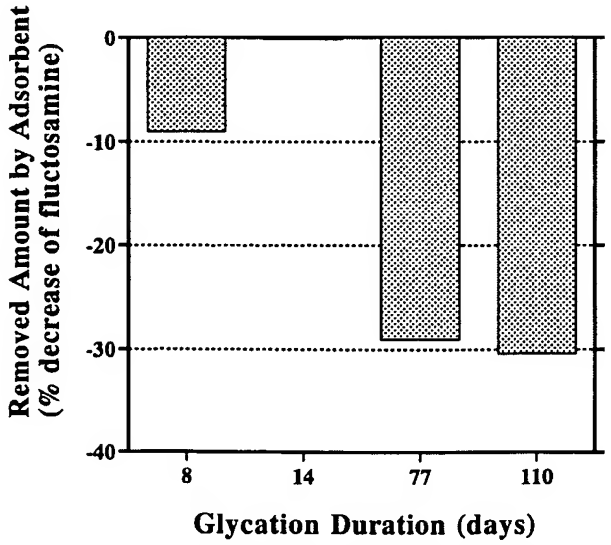

FIG. 1. Shown is the relationship between glycation duration and the decrease in fructosamine concentration by hydrazine-conjugated cellulose.

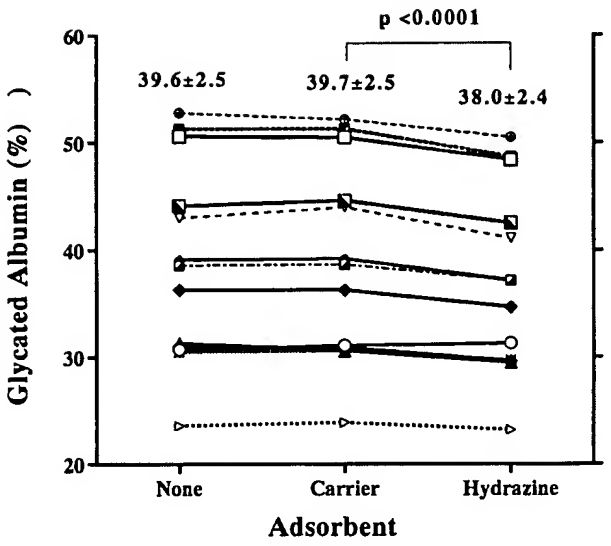

FIG. 2. The comparison is of glycated albumin concentration among samples before treatment (none), after incubation with cellulose alone (carrier), and other incubation with $NHNH_2$-conjugated cellulose (hydrazine).

DISCUSSION

The plausible pathogenic mechanism of diabetic nephropathy is AGE assisted bridge formation between proteins in the extracellular matrix (8) or the changes in the functions of glomerular mesangial cells by AGE (9). The increase in type IV collagen production by AGE is prohibited by administration of aminoguanidine (10). The production of type IV collagen is also increased by incubation with Amadori substances. The monoclonal antibody to Amadori substances reduced proteinuria in association with the inhibition of type VI collagen production (11). Thus, the Amadori substances play important roles in the development of complications resulting from diabetes mellitus. The goal of this study was to devise an apheresis method for the selective adsorption of Amadori substances.

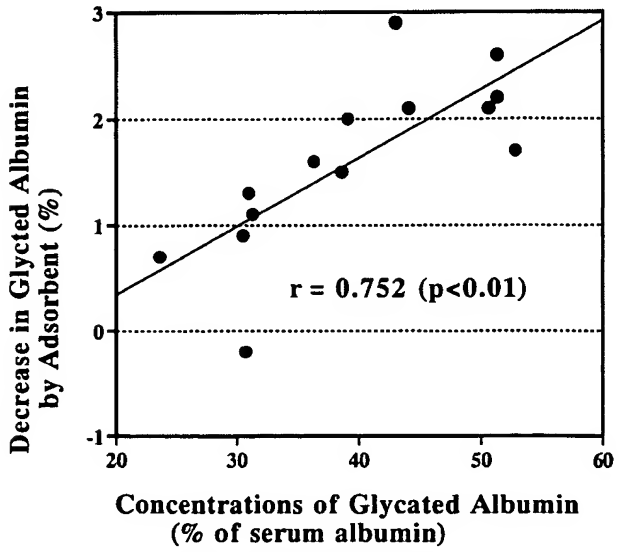

FIG. 3. The graph shows the relationship between the concentrations of glycated albumin and the adsorbed amounts of glycated albumin.

Previously, Ogino and Tani (5) had examined the binding activity of several compounds in which an immobilized amino group was used as a ligand of Amadori products. They showed that model compounds of Amadori products containing a ketoamino group were adsorbed more intensely with hydrazine-conjugated cellulose than with the other adsorbents (5). The current study suggested that artificially glycated proteins with longer glycation periods were more completely adsorbed with hydrazine-conjugated cellulose. Moreover, the adsorbent had greater affinities for the glycated albumin in human serum samples with a higher glycation percent. These results indicate that hydrazine-conjugated cellulose can specifically adsorb the highly glycated proteins. The complications due to diabetes mellitus are related to long-standing glycation of proteins. The proteins with a greater grade of glycation seem to be more pathogenic. The results of the current study provide a clue for the development of an adsorbent for highly glycated protein. However, the adsorbed portion of glycated albumin was only 1.7% in human serum samples in contrast to over 20% in the artificially glycated protein. This discrepancy may be related to the presence of substances in the serum that competitively inhibit the binding of glycated albumin. The further modification of the adsorbent may improve the efficiency of adsorption.

Several hydrazine derivatives have toxic side effects, including carcinogenesis (12). Other studies have shown that some hydrazine derivatives have antitumor activity against murine L1210 leukemia and were capable of producing "cures" of mice bearing this tumor (13). In the current study, hydrazine was bound to cellulose, and free hydrazine was discarded by washing with PBS solution. Even if the hydrazine-bound cellulose is used as an adsorbent of glycated proteins, the amount of free hydrazine which will enter the body seems to be small. Therefore, the carcinogenesis of hydrazine is not a critical factor for clinical application of hydrazine-conjugated cellulose.

In conclusion, hydrazine-conjugated cellulose has a higher affinity for albumin with greater glycation and may provide a clue for the development of an adsorbent for highly glycated protein with pathogenic significance.

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Advanced Glycation and Lipoxidation End Products: Role of Reactive Carbonyl Compounds Generated during Carbohydrate and Lipid Metabolism

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The uremic syndrome is usually attributed to the retention of a variety of compounds as a result of a deficient renal clearance. These retention solutes are taken to induce biochemical disorders characteristic of uremic complications. However, as recently noted by Vanholder and De Smet (1), only a few of them have an established role as uremic toxins.

Most studies on uremic toxins have focused on disorders of enzymatic biochemistry and/or on toxins that are the result of enzymatic biochemistry. In this review, we concentrate on another aspect of uremic toxicity, related to nonenzymatic biochemistry of proteins. We investigate the causal role of various reactive carbonyl compounds (RCO) accumulating in the serum; postulate the existence of "carbonyl stress" in uremia and analyze its clinical consequences; and, finally, discuss therapeutic perspectives.

Accumulation of Advanced Glycation End Products/Advanced Lipoxidation End Products in Uremia

The advanced glycation of proteins has been initially investigated by food and nutrition biochemists (2). The Maillard reaction, a nonenzymatic process, is initiated when proteins are exposed to glucose or other carbohydrates. It generates first reversible Schiff base adducts and subsequently more stable Amadori rearrangement products. Through a series of oxidative and nonoxidative reactions, it eventually yields the irreversible advanced glycation end products (AGE) linked with amino groups, *e.g.*, lysine residues, of several proteins.

In human pathology, irreversible advanced glycation of proteins is a part of the ageing process. It is markedly amplified in diabetes, as a consequence of hyperglycemia: AGE levels are indeed correlated with those of fructoselysine, taken as a surrogate marker of prevailing plasma glucose concentration (3–5). Of interest, they are also correlated with the severity of diabetic complications, a finding supporting their clinical relevance (6–8).

The subsequent discovery that AGE also accumulate in uremic patients was surprising because most uremic individuals have normal blood glucose levels. Sensitive and specific chemical methods including HPLC (9) and gas chromatography/mass spectrometry (GC/MS) (10) were developed to quantify AGE, such as pentosidine (11) and carboxymethyllysine (CML) (12). The levels of these two AGE adducts in plasma proteins of hemodialysis patients proved markedly higher than in control or diabetic subjects (13,14). Other AGE adducts also accumulate in uremia, such as glyoxal-lysine dimer, methylglyoxal-lysine dimer, and imidazolone (15).

Among dialysis patients, those with diabetes and those without had similar plasma pentosidine and CML levels (13,14). In contrast to nonuremic diabetic patients, neither pentosidine nor CML correlated with fructoselysine levels in uremic subjects. Thus, it became clear that factor(s) other than hyperglycemia are critical for AGE formation in uremia. The fact that more than 90% of plasma pentosidine and CML are albumin adducts (13,14) suggests that its accumulation does not result from a decreased renal clearance of AGE-modified proteins.

The second approach to irreversible protein modification in uremia derives from studies of lipid metabolism, especially lipid peroxidation. Proteins are modified not only by carbohydrates but also by lipids (16). For instance, proteins modified by malondialdehyde, which is derived from the oxidation of polyunsaturated fatty acids such as arachidonate, accumulate in hemodialysis patients (14). Malondialdehyde as well as other lipid peroxidation product modified proteins are called advanced lipoxidation end products (ALE) (17).

Uremia is thus characterized by irreversible nonenzymatic protein modifications by carbohydrates or lipids, *i.e.*, AGE/ALE (Table 1). Of note, the levels of AGE and ALE rise concomitantly in uremic serum: Plasma CML, an AGE species, is highly correlated with plasma malonyldialdehyde-lysine, an ALE species, in patients on chronic hemodialysis (14). This observation points to a common cause in the genesis of AGE/

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Table 1. Accumulation of AGE/ALE in hemodialysis patients[a]

AGE/ALE	Precursor(s)	Reference
Pentosidine	Carbohydrate	13, 18
Carboxymethyllysine	Carbohydrate, lipid, amino acid	14
Malondialdehydelysine	Lipid	14
Imidazolone	Carbohydrate	19
Glyoxal-lysine dimer	Carbohydrate, lipid, amino acid	15
Methylglyoxal-lysine dimer	Carbohydrate, lipid, amino acid	15

[a] AGE, advanced glycation end product; ALE, advanced lipoxidation end product.

ALE. This conclusion is further supported by a recent report that, in the skin of renal failure patients, lipid peroxidation and advanced glycation of matrix collagen increase in close relation to each other (20).

Accumulation of Reactive Carbonyl Compounds in Uremia: Carbonyl Stress

Both AGE and ALE are formed by carbonyl amine chemistry between protein residues and reactive carbonyl compounds (RCO) (17). These RCO are constantly produced by the metabolism of carbohydrates, lipids, and amino acids, all of which are abundantly present throughout the body. RCO, *e.g.*, glyoxal, methylglyoxal, arabinose, glycoaldehyde, 3-deoxyglucosone, and dehydroascorbate, are formed from carbohydrates and ascorbate (21–25). They react nonenzymatically with protein amino groups and eventually yield AGE, *e.g.*, CML, pentosidine, pyrraline, imidazolone, glyoxal-lysine dimer, and methylglyoxal-lysine dimer. Similarly, RCO, *e.g.*, glyoxal, malondialdehyde, hydroxynonenal, and acrolein, are generated by lipid peroxidation of polyunsaturated fatty acids (16,26). In addition, RCO, such as glyoxal, methylglyoxal, acrolein, and glycoaldehyde, are produced during the myeloperoxidase catalyzed metabolism of amino acids (27). These RCO react with proteins and form ALE as well as AGE.

Could the raised levels of AGE and ALE in uremia accrue from an accumulation of carbohydrate- and lipid-derived RCO? Total RCO have been measured in uremic plasma with the 2,4-dinitrophenylhydrazine (DNPH) method. DNPH is known to combine with RCO and to yield hydrazones. The yield of hydrazone is indeed several times higher in uremic than in normal plasma (28). The accumulation of total as well as of individual RCO has also been documented by several groups (17,28–31). Table 2 summarizes the various RCO thus far found to be raised in uremic plasma.

The production of low molecular weight AGE precursors in uremic plasma has also been demonstrated by *in vitro* incubation experiments (28). Plasma samples were incubated under air for several weeks while the generation of pentosidine in the medium was monitored. Protein-linked pentosidine levels rise much more in uremic than in control plasma. Most precursors of this newly formed pentosidine have a molecular weight below 5000 Da. Indeed, when plasma is ultrafiltrated through a filter with a 5000 Da cutoff, the difference in pentosidine generation between uremic and control plasma ultrafiltrate is sustained. This conclusion is further supported by the observation that the pentosidine yield is higher in pre- than in postdialysis plasma samples. Finally, addition of inhibitors of the carbonyl amine reaction, such as aminoguanidine or 2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195), represses the production of pentosidine, pointing to the RCO nature of the pentosidine precursor(s). Taken together, these results confirm the accumulation of RCO in uremic plasma and suggest that they are indeed precursors of AGE.

In vivo pentosidine levels in plasma are correlated with the level of *in vitro*-generated pentosidine after incubation of the same plasma samples (28). This correlation suggests that the prevailing plasma pentosidine level mirrors the level of its RCO precursor(s) and might be used as a marker of their accumulation.

The accumulation in uremic plasma of various RCO derived from either carbohydrates or lipids and the subsequent carbonyl modification of proteins suggest that chronic uremia may be characterized as a state of carbonyl stress (17). Under

Table 2. Accumulation of RCO in hemodialysis patients[a]

RCO	Precursor(s)	Reference
3-Deoxyglucosone	Carbohydrate	29
Dehydroascorbate	Ascorbic acid	31
Glyoxal	Carbohydrate, lipid, amino acid	30
Methylglyoxal	Carbohydrate, lipid, amino acid	30
Malondialdehyde	Lipid	14
Arabinose	Carbohydrate	Miyata *et al.*, unpublished observation

[a] RCO, reactive carbonyl compound.

carbonyl stress, not only AGE derived from carbohydrates, but also ALE derived from lipids accumulate in parallel in plasma as well as in tissue proteins.

Causes of Uremic Carbonyl Stress

Two competing but not mutually exclusive hypotheses should be considered to account for the uremic carbonyl stress: an increased generation or a decreased removal (detoxification or clearance) of RCO.

Oxidative Stress

Production of RCO is known to be increased by oxidative stress. Several reports point to an increased oxidative stress in uremia, characterized by an augmented production of oxidants and a decreased level of antioxidants. The evidence includes increased serum ratios of oxidized to reduced ascorbate (31), glutathione (32), and albumin (33); increased serum levels of advanced oxidation protein products (34) and of protein carbonyls (35); decreased serum activity of glutathione-dependent enzymes (36,37); increased lipid peroxidation products (14); and accumulation of dialyzable oxidants (38). The uremic oxidative stress might be further worsened by some modalities of renal replacement therapy: The hemodialysis treatment activates complement and leukocytes, which release reactive oxygen species (39,40).

The oxidative stress modifies proteins either directly through the oxidation of amino acids by reactive oxygen species (41) or indirectly by an increased generation of RCO (17). Carbohydrates and lipids targeted by reactive oxygen species yield, as mentioned earlier, increased amounts of RCO involved eventually in the formation of AGE and ALE ($k_1 + k_3$ in Figure 1). A causal role of the oxidative stress in AGE and ALE formation is supported by the correlation existing in uremic serum between pentosidine and oxidative markers such as dehydroascorbate (31) and advanced oxidation protein products (34).

The relevance of these abnormalities to intracellular events remains to be thoroughly documented. The detection of AGE and ALE by immunohistochemistry in vascular and renal tissues suggests that a local oxidative stress may contribute to pathologic lesions, such as fatty streak and thickened intima of arterial walls in atherosclerosis (42,43) and expanded mesangial area and nodular lesions in diabetic nephropathy (44,45).

The oxidative stress hypothesis, however, is not wholly satisfactory. Indeed, RCO such as 3-deoxyglucosone and methylglyoxal are derived from nonoxidative chemistry. 3-Deoxyglucosone is formed nonoxidatively by rearrangement and decomposition of Amadori compounds or by anaerobic metabolic reactions leading to formation of fructose-3-phosphate, which decomposes spontaneously to 3-deoxyglucosone (24). The more reactive RCO, methylglyoxal, is also formed during anaerobic metabolism of acetone and amino acids (22). Both RCO react with proteins and form AGE. Raised levels of 3-deoxyglucosone and methylglyoxal and of their protein adducts in uremia (15,29) demonstrate that nonoxidative chemistry is also involved in the generation of the carbonyl stress ($k_4 + k_5$).

Impaired RCO Detoxification

The rise in RCO in uremia might also be accounted for by a decreased removal. RCO are detoxified by several enzymatic pathways, such as aldose reductase, aldehyde dehydrogenases, and the glyoxalase pathway (22). Redox coenzymes, reduced glutathione (GSH) and nicotinamide adenine denucleotide (phosphate) (NAD(P)H), contribute to their activity. RCO such as methylglyoxal and glyoxal react reversibly with the thiol

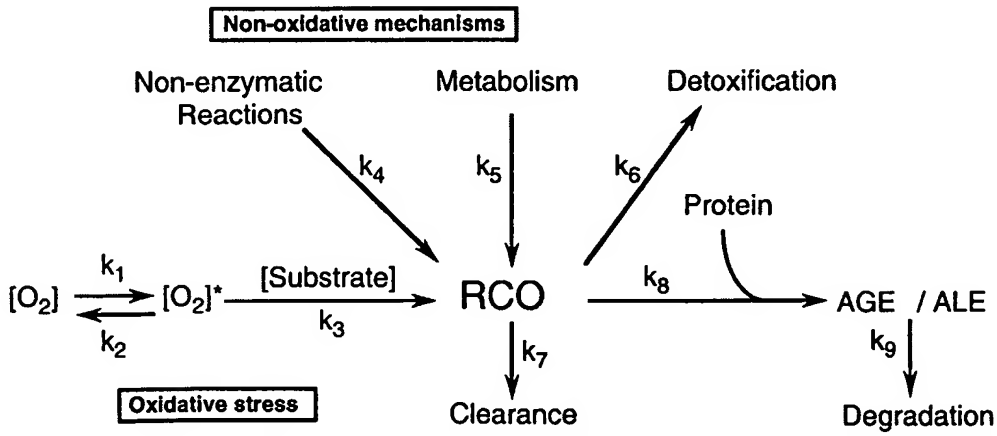

Figure 1. Routes to generation and detoxification of reactive carbonyl compounds (RCO). The level of oxidative stress is depicted as the balance between the overall rate of formation of reactive oxygen species (ROS) ($k_1 \times [O_2]$) and the rate of their inactivation by antioxidant defenses ($k_2 \times [O_2]^*$). ROS react with carbohydrates, polyunsaturated fatty acids, or amino acids ($k_3 \times [O_2]^* \times$ [Substrate]) to yield RCO. RCO are also formed by nonenzymatic reactions (k_4: *e.g.*, 3-deoxyglucosone) and by anaerobic metabolism (k_5: *e.g.*, methylglyoxal). RCO are detoxified ($k_6 \times$ [RCO]) by a variety of metabolic pathways (*e.g.*, the glyoxalase pathway) or probably cleared from the body (k_7). The RCO subsequently react with protein ($k_8 \times$ [RCO] $\times$ [Proteins]) to form advanced glycation end products (AGE)/advanced lipoxidation end products (ALE). The increase in RCO in uremia results from an increase in their production ($k_3 + k_4 + k_5$) and/or a decrease in their removal ($k_6 + k_7$). The AGE/ALE formation depends on the balance between their rates of formation (k_8) and degradation (k_9).

group of glutathione and are subsequently detoxified by glyoxalases I and II into lactate and glutathione. NAD(P)H replenishes glutathione by increasing the activity of glutathione reductase. Decreased levels of glutathione and NAD(P)H can therefore result in augmented levels of a wide range of RCO (k_6).

It is of interest to know in this context that the glutathione concentration in red blood cells and the serum activity of glutathione-dependent enzymes are significantly reduced in uremic patients (32,36,37). The hypothesis that a decreased thiol storage capacity contributes to the accumulation of RCO in uremia is supported by recent evidence. Glutathione peroxidase activity is indeed inversely correlated with the pentosidine levels in the plasma of hemodialysis patients (37). Of course, other as yet unexplored enzymatic mechanisms might contribute to a decreased removal of RCO and thus to the uremic carbonyl stress.

It should be pointed out that the decreased thiol concentration in uremia might reflect its consumption during detoxification of reactive oxygen species generated under uremia-associated oxidative stress. Still, as yet unproven in uremia, a nonoxidative pathway to decrease thiol concentration has been recognized in diabetes. The polyol pathway is activated by hyperglycemia and consumes NAD(P)H for the reduction of glucose to sorbitol, a process catalyzed by aldose reductase (46). The decrease of NAD(P)H availability for GSH reductase lowers GSH levels. This process depends on glucose concentration but is independent of oxidative stress. It remains to be seen whether the decrease of thiol concentration in uremia derives only from oxidative stress or from other nonoxidative pathways.

Decreased Glomerular Filtration of RCO

RCO derived from both oxidative and nonoxidative chemistry of both carbohydrates and lipids have a rather low molecular weight. A decreased renal clearance may contribute to the uremic carbonyl stress (k_7). In fact, the pentosidine level in both uremic and diabetic patients is strongly influenced by residual renal function (8,13,47). If pentosidine levels are taken as markers of RCO precursors (28), these data point to a significant role of the failing kidney in raising plasma RCO levels.

Implications of Carbonyl Stress

The consequences of the uremic carbonyl stress and its attendant AGE and ALE modification of proteins are discussed below.

Cellular Effects of RCO

Several lines of evidence suggest that RCO interfere with various cellular functions independently of their effect on AGE and ALE modification of proteins. RCO are biologically active, initiate a variety of cellular responses, and induce structural and functional alterations of proteins (17,48).

For example, renal failure is associated with resistance to the action of calcitriol (1,25-dihydroxyvitamin D) (49), which is partly attributed to the inhibition by unknown uremic toxins of the interaction between the vitamin D receptor and vitamin D response elements (50). Subsequently, Patel and coworkers (51) demonstrated that RCO capable of Schiff base formation with lysine residues of the vitamin D receptor inhibit its interaction with the vitamin D response element.

In another model, exposure *in vitro* of cultured mesothelial and endothelial cells to methylglyoxal increases mRNA and protein synthesis of vascular endothelial growth factor (VEGF) (52). VEGF also increases *in vivo* in the peritoneal tissue of rats given repeated intraperitoneal loads of methylglyoxal (52).

Carbonyl stress also influences the intracellular signaling by multiple pathways. First, AGE, upon interaction with the receptor for AGE, trigger the signaling involving ras pathway (53). Second, exposure of fibroblasts to glyoxal activates protein kinases such as c-Src and increases intracellular tyrosine phosphorylation of several cellular proteins (54). This effect is mediated by the formation of Schiff base on cell surface protein since it is prevented by an inhibitor of the carbonyl amine reaction, OPB-9195 (54). Third, hydroxynonenal causes a capping of epidermal growth factor (EGF) receptor on the cell surface, mimics the effect of EGF on the downstream signaling pathways that involve mitogen-activated protein kinases, and contributes to oxidative stress-induced apoptotic cell death (55). Fourth, hydroxynonenal also triggers oxidative stress-induced apoptotic cell death by activating caspase-3 through a Fas-independent but GSH-dependent redox pathway (56). Finally, methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase (57).

AGE and ALE Biologic Effects

Carbonyl stress is also implicated in the development of several uremic complications. Dialysis-related amyloidosis is a serious bone and joint destruction associated with chronic renal failure (58). The cause of β_2-microglobulin amyloid fibril deposition is not fully elucidated. The role of elevated plasma β_2-microglobulin levels is disputed because no difference has been found between dialysis patients with and those without clinical evidence of complications (59). Efforts have therefore been directed toward identification of chemical modifications of β_2-microglobulin. Although these studies are incomplete, immunohistochemical and chemical analyses indicate that β_2-microglobulin amyloid deposits are modified by carbonyl stress. Long-lived β_2-microglobulin amyloid plaques react with anti-AGE (CML, pentosidine, and imidazolone) and anti-ALE (malondialdehyde-lysine and hydroxynonenal-protein adduct), as well as anti-receptor for AGE antibodies (60–65). β_2-Microglobulin amyloid fibrils isolated from plasma and urine of uremic patients also reacts with these antibodies (9,60).

Atherosclerosis is a major complication of chronic renal failure. The levels of AGE in randomly collected arterial tissues are higher in dialysis patients than in healthy subjects (66). AGE and ALE are detectable by immunohistochemistry in the fatty streak and in the thickened neointima (43). The staining patterns of AGE and ALE correspond with that for protein carbonyls, a biomarker of oxidative protein damage (42). The formation of AGE/ALE in vascular lesions is not

specific to uremia, but might be a phenomenon common to most, if not all, types of vascular damage, regardless of whether the vascular injury is caused by metabolic or mechanical factors: AGE/ALE are identified in vascular lesions not only in uremic patients but also in aged and diabetic subjects (42), and in nonuremic rats whose carotid artery has been injured by a balloon (43). Colocalization of AGE/ALE and protein carbonyls in the vascular tissue indicates a wide range of chemical modifications in vascular matrix proteins.

The quality of the peritoneal membrane deteriorates progressively with peritoneal dialysis (PD) duration (67). Membrane alterations, characterized by interstitial fibrosis, disappearance of mesothelial cells, vascular wall thickening, vasodilation, and increased angiogenesis develop progressively, together with changes in permeability characteristics. The glucose content of PD fluid has been incriminated. Glucose is degraded during heat sterilization into a variety of RCO, such as methylglyoxal, glyoxal, and 3-deoxyglucosone (68–70). These RCO might play a role in the development of AGE and ALE accumulating in the mesothelial layers and in the vascular walls of the peritoneum (70,71). Interestingly, AGE such as CML and pentosidine colocalize in the peritoneum with VEGF (52,72). These findings support the implication of peritoneal modification by RCO in the liberation of VEGF, a potent factor enhancing vasodilation, vascular permeability, angiogenesis, and nitric oxide synthase production (73), all of which may contribute to peritoneal membrane deterioration.

In these three examples, it remains to be seen whether the presence of AGE and ALE merely result from the long-term accumulation of protein modifications and is therefore an inert surrogate marker for carbonyl stress, or, alternatively, whether it plays an active role in the pathogenesis of these complications. Recent studies support the latter hypothesis. AGE- and ALE-modified proteins prepared *in vitro* initiate a range of inflammatory responses, including stimulation of monocyte chemotaxis (74,75), secretion of inflammatory cytokines from macrophages (74–76), stimulation of collagenase secretion from synovial cells (74), stimulation of osteoclast-induced bone resorption (77), proliferation of vascular smooth muscle cells (78), stimulation of aggregation of platelets (79), stimulation of VEGF (80) and platelet-derived growth factor (81) production from endothelial cells, induction of insulin-like growth factor I from monocytes (82), inhibition of antibacterial activity of lysozyme and lactoferrin (83), and quenching nitric oxide activity (84).

Therapeutic Approaches

Carbonyl stress is clearly implicated in the development of several uremic complications. Its manipulation should provide new therapeutic insights. Among them are redox modulation, the use of inhibitors of carbonyl amine chemistry, and the improvement of dialyzer membrane biocompatibility.

Redox Therapy

The decrease in thiol concentration impairs the detoxification of RCO and potentiates the formation of AGE and ALE. Repletion of thiol might prove possible and useful by supplementation of glutathione, *N*-acetylcysteine, or cysteine. Addition of these thiol compounds in both uremic and normal plasma as well as in glucose-based PD fluid lowers the generation of AGE after incubation *in vitro* (our unpublished observation). Several other compounds may also prove helpful. Vitamin E and ubiquinol relieve the demands on the activity of glutathione. Lipoic acid is reduced by mitochondrial dehydrogenases to dihydrolipoate, which subsequently reacts with reactive oxygen species and replenishes glutathione (85). Inhibitors of aldose reductase are other candidates to replenish glutathione, as they prevent the polyol pathway activation and replenish NAD(P)H and glutathione available for both aldose reductase and glutathione reductase (86). Administration of lipoic acid (87) or of the aldose reductase inhibitor Statil (88) to rats with streptozotocin-induced diabetes increases thiol concentration and/or decreases methylglyoxal in tissues and blood.

Carbonyl Stress Inhibitor

AGE/ALE are formed by carbonyl amine chemistry between RCO and proteins. Trapping of RCO with substances such as aminoguanidine (89) and OPB-9195 (90) should inhibit the formation of AGE and ALE. Indeed, we have demonstrated that both compounds inhibit the *in vitro* formation of AGE from a variety of individual precursors such as ribose, glucose, and ascorbate, as well as that of ALE, malondialdehyde-lysine, and hydroxynonenal-protein adduct from arachidonate (91). Pentosidine generation in uremic plasma (28) and in glucose-rich PD fluid (91) incubated for 4 wk is also inhibited by aminoguanidine and OPB-9195.

On a molar basis, OPB-9195 is more effective than aminoguanidine because the latter's hydrazine nitrogen atom has a decreased nucleophilicity due to the proximity of the guanidinium cation. OPB-9195 as well as aminoguanidine might act by an antioxidative mechanism inhibiting the production of RCO from the various substrates. Alternatively, it may trap the available RCO and thus prevent AGE formation. The observation that OPB-9195 markedly decreases *in vitro* the level of RCO present in glucose-based PD fluid (91) strongly supports the latter hypothesis. Trapping may result from the reaction of the hydrazine nitrogen atom of aminoguanidine and OPB-9195 with carbonyl groups, leading to the eventual formation of hydrazones.

Interestingly, OPB-9195 corrects several biologic effects of RCO. In murine thymocytes and fibroblasts, it inhibits the phosphorylation of tyrosine residues of a number of intracellular proteins induced by glyoxal (54). Given to Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats, a model of non-insulin-dependent diabetes mellitus, OPB-9195 reduces urinary albumin excretion and improves the morphology of glomeruli (90). Furthermore, oral administration of OPB-9195 to rats, after balloon injury of their carotid arteries, effectively reduces neointima proliferation in arterial walls, an early and major step in the development of atherosclerotic lesions (43).

The development of less toxic and more specific carbonyl stress inhibitors should prove an important new therapeutic

avenue. Such compounds immobilized in cartridges might enhance extraction of RCO from blood during dialysis therapy.

Membrane Biocompatibility

An unexpected observation has yielded an additional therapeutic insight. A cross-sectional and longitudinal study of dialyzed patients has shown that both protein-linked and free pentosidine levels are lower in the plasma of patients given polysulfone dialysis than in those treated with several other membranes (47,92), including the equally biocompatible toward the complement and leukocyte system high-flux AN69 membrane and the less biocompatible low-flux cuprophane membrane. Pentosidine levels are lower in Belgian, Japanese, and German patients given dialysis with polysulfone membranes produced by two different companies.

There is at present no obvious explanation for these results. The effect is unrelated to the porosity or to the clearing ability of the membrane for pentosidine: Hemodialysis itself does not modify protein-linked pentosidine levels (more than 90% of pentosidine is linked to nondiffusible albumin), and the clearance of free pentosidine (379 Da) during a single dialysis session is similar for all membranes (18). The lower level of pentosidine probably reflects a lower generation of RCO, precursors of pentosidine through an as yet undefined mechanism. Much research remains to be done to demonstrate the clinical relevance of these observations.

Until recently, membrane biocompatibility has been discussed in relation to acute, enzymatic biochemistry, such as leukocyte and complement activation, and production of cytokines. However, long-term, nonenzymatic biochemistry may also be equally important in terms of membrane biocompatibility.

Conclusion

Research on AGE and ALE has led to new insights in nonenzymatic biochemistry in renal failure. It has revealed the accumulation of RCO derived from carbohydrates and lipids, the so-called carbonyl stress. Carbonyl stress alters the structure and function of cellular and matrix proteins and might underlie the development of long-term complications. It may prove amenable to therapeutic interventions. Carbonyl stress should therefore be considered a major contributor to uremic toxicity.

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PERSPECTIVES IN RENAL MEDICINE

Toward better dialysis compatibility: Advances in the biochemistry and pathophysiology of the peritoneal membranes

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Toward better dialysis compatibility: Advances in the biochemistry and pathophysiology of the peritoneal membranes. Peritoneal dialysis (PD) has modified our concept of the peritoneal membrane, which is now a topic of active research. Peritoneal solute transport progressively increases with time on PD, enhances the dissipation of the osmotic gradient and, eventually, reduces ultrafiltration capacity. The causes of peritoneal membrane failure remain elusive. Recurrent episodes of peritonitis are not a prerequisite for the development of ultrafiltration failure. Functionally, the changes of the failing peritoneal membrane are best described as an increased functional area of exchange for small solutes between blood and dialysate. Histologically, these events are associated with vascular proliferation and structural changes of pre-existing vessels. Gathered evidence, including information on the composition of peritoneal cavity fluids and its dependence on the uremic environment, have cast a new light on the molecular mechanisms of decline in peritoneal membrane function. Chronic uremia per se modifies the peritoneal membrane and increases the functional area of exchange for small solutes. Biochemical alterations in the peritoneum inherent to uremia might be, at least in part, accounted for by severe reactive carbonyl compounds overload originating both from uremic circulation and PD fluid ("peritoneal carbonyl stress"). The molecular events associated with long-term PD are similar but more severe than those present in chronic uremia without PD, including modifications of nitric oxide synthase (NOS) and angiogenic growth factors expression, and advanced glycation and lipoxidation of the peritoneal proteins. This review focuses on reactive carbonyls and their association with a number of molecular changes observed in peritoneal tissues. This hypothetical approach will require further testing. Nevertheless, the insights gained on the peritoneal membrane offer a new paradigm to assess the effect of uremic toxins on serosal membranes. Furthermore, the progresses made in the dissection of the molecular events leading to peritoneal membrane failure open new avenues to develop safe, more biocompatible peritoneal dialysis technologies.

Key words: ultrafiltration failure, effective peritoneal surface area, angiogenesis, nitric oxide, vascular endothelial growth factor, carbonyl stress, advanced glycation end products, glucose degradation products.

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Peritoneal dialysis (PD) is now an established alternative in the treatment of end-stage renal failure. It is utilized in approximately 15% of the dialysis patients in the developed world [1]. It has proven to be better than hemodialysis, especially in its protection of residual renal function [2] and in the lower overall cost to society [3]. Its use has been limited by the morbidity associated with acute peritonitis episodes, a problem recently overcome by newer technical devices and better antibiotic management. The major remaining problem is the progressive deterioration of the peritoneal membrane structure and function [4], which curtails its use in approximately 50% of PD patients within five years [5]. Cross-sectional and longitudinal studies have shown that peritoneal solute transport progressively increases with time on PD, enhances the dissipation of the osmotic gradient, and eventually reduces ultrafiltration capacity [6–8]. This increased small solute transport rate mainly reflects the peritoneal vascular surface area [4]. A raised peritoneal membrane permeability is associated not only with technique failure, but also with increased mortality and morbidity in PD patients [9].

The causes of peritoneal membrane failure remain elusive. Although recurrent episodes of peritonitis with associate inflammatory changes damage the peritoneal membrane over time [10], they are by no means a prerequisite for the development of ultrafiltration failure [4, 11]. Recent studies have cast a new light on the molecular mechanisms of decline in peritoneal membrane function during PD therapy. This review summarizes a few of them, delineates the causal role of reactive carbonyl compounds (RCOs) within the peritoneal cavity whether they originate from conventional heat-sterilized glucose PD fluid or from the uremic circulation, provides a hy-

pothesis on the molecular events leading to alterations of the peritoneal membrane transport and, finally, discusses therapeutic perspectives toward more effective and biomedically more suitable PD technologies.

THE PERITONEAL MEMBRANE: A UNIQUE AND EFFICIENT EXCHANGE SURFACE BETWEEN BLOOD AND DIALYSATE

The histology of the peritoneal membrane has been clearly described to include the mesothelium, interstitial space, and blood microvessels. Under normal physiological conditions, the mesothelial layer [4, 12, 13] and interstitial tissue [14, 15] are not thought to be important barriers at least for small solute transport. By contrast, the peritoneal vascular walls, mainly through their endothelium, are the main obstacles to small solute transport during PD [12, 13].

In standard PD using glucose as the osmotic agent, the peritoneal membrane exchange potential has been evaluated by clinical tools such as the peritoneal equilibrium test (PET) and sodium sieving. Its efficiency relies on the osmotic gradient between peritoneal fluid and plasma as it allows water influx, the diffusion of small uremic solutes evaluated by urea and creatinine, and the counter diffusion of glucose.

The results of the PET and sodium sieving have been modeled and a series of pores have been described [16]: ultrasmall pores (<0.3 nm) for water, small pores (4 to 5 nm) for water and smaller solutes such as urea and glucose, and large pores (>15 nm) for all the former constituents and large solutes. The fact that 50% of the ultrafiltration during 2.27 or 3.86% dwell occurs through the ultrasmall pores illustrates their major clinical importance in PD patients [17].

Recent studies have identified a number of molecules that functionally mediate the peritoneal membrane characteristics. The water channel aquaporin-1 (AQP-1) is located in the apical and basolateral membranes of endothelial cells lining non-fenestrated capillaries in numerous tissues including the peritoneum [18]. Several lines of evidence demonstrate that AQP-1 is the molecular counterpart of the ultrasmall pore of the peritoneal membrane [19, 20]. The small and large pores are not yet specifically identified. Additional molecules include nitric oxide (NO)/nitric oxide synthase (NOS) [21, 22], vascular endothelial growth factor (VEGF) [23], and basic fibroblast growth factor (FGF2) [24]. Both VEGF and FGF2 are instrumental in vascular proliferation and neo-angiogenesis. In addition, FGF2 mediates fibrotic changes of the interstitium and smooth muscle cell proliferation [25].

DOES UREMIA MODIFY THE PERITONEAL MEMBRANE REGARDLESS OF PD?

Until very recently it was generally accepted that the peritoneal membrane was unaffected by uremia, although an increased permeability had been already documented [26]. In a rat model of chronic uremia, Combet et al observed structural modifications including submesothelial and perivascular fibrosis, and angiogenesis [27]. These changes were matched by functional modifications characterized by an augmented permeability for small solutes. Several potential mediators identified thus far include VEGF and FGF2 expression, followed by endothelial NOS (eNOS) and neuronal NOS (nNOS), as they were transiently up-regulated after subtotal nephrectomy. The severity of these various uremia-induced alterations correlated with the degree of renal failure. Of note, a similar increase in FGF2 expression has been observed in the peritoneum of uremic patients, prior to the initiation of dialysis (Ferrier ML and Devuyst O, unpublished observation).

In addition to changes in some factors already present in the normal rat peritoneum, uremia is associated with vascular deposits of N^{ε}-carboxymethyllysine (CML) and pentosidine [27], two well-known epitopes generated by advanced glycation, that is, advanced glycation end products (AGEs). Their localization coincides with that of overexpressed VEGF and FGF2. In contrast to the rat model, in humans AGE modification of peritoneal proteins appears, at best, faint [28, 29].

The peritoneal membrane structural alterations detected in the uremic rat model have been supported in humans [30, 31]. Even in non-dialyzed uremic patients, submesothelial fibrosis is present and the density of vessels increased, as compared to non-uremic controls. Altogether, these data suggest that biochemical alterations inherent to uremia *per se* modify the peritoneum prior to the use of PD, and thus provide a paradigm to better understand the modification of the several serosal membranes in uremia.

CHANGES IN THE PERITONEAL MEMBRANE DURING PD

The permeability characteristics of the peritoneal membrane are progressively modified during PD, imposing occasional switching of the patient from PD to hemodialysis.

Functional analysis

The most striking functional characteristics of peritoneal membrane failure are detected in patients on chronic PD by the PET test and a measure of sodium sieving [4, 6–8]. These characteristics include a loss of free water permeability identified by the disappearance of sodium sieving, an increased transport rate of small solutes (urea, creatinine and glucose), with an attendant

enhanced dissipation of the glucose-dependent osmotic gradient across the peritoneal membrane and the loss of ultrafiltration capacity.

The changes in the functional characteristics of the failing peritoneal membrane are best described as an increased functional area of exchange for small solutes between blood and dialysate, the so-called "effective peritoneal surface area" (EPSA) [4, 12]. They rely on enhanced angiogenesis (described below) and, possibly, vasodilation of peritoneal vessels. The increased transport of small solutes stands in contrast with the impaired water transport, witnessed by the fading sodium sieving.

Histological analysis

Histopathologic studies have revealed that prolonged PD is associated with profound modifications of the peritoneal membrane. First, an increased density of blood vessels, documented in a rat model of long-term PD [32], has been recently confirmed in humans [31, 33, 34]. Quantification of factor VIII immunoreactivity in peritoneal sections from long-term PD patients, showed a 2.5-fold increase in the density of stained vessels in long-term PD patients as compared to non-uremic controls [33]. The endothelial area also was increased in the PD patients [33]. Thus, PD progressively increases the EPSA as the result of vascular proliferation and, possibly, of a vasodilation of pre-existing vessels. Interstitial fibrosis and mesothelial alterations have been reported in addition to the vascular changes [30, 31, 34].

Molecular biological analysis

In humans, the molecular events associated with long-term PD and peritoneal membrane failure are similar but more severe than those present in chronic uremia without PD. They include up-regulation of eNOS, overexpression of VEGF and FGF2, and advanced glycation of peritoneal proteins [33]. In contrast, the expression of AQP1, the pore involved in water transport, remains apparently unaffected [35].

The up-regulation of VEGF and FGF2 fits with an enhanced angiogenesis and the interstitial fibrosis observed on histological analysis: its determinants are discussed later in this article. The increased NOS activity is also of considerable interest as NO has been shown to be clinically relevant [36–38]. Under some circumstances, the NO donor, nitroprusside, enhances both the EPSA and the intrinsic permeability of the membrane both in animal models and in PD patients, whereas NOS inhibitors such as L-NAME have the reverse effect. These effects of NO are in good agreement with its previously described activities in other systems [39–42]: NO augments vascular permeability and modulates the expression and activity of VEGF. Furthermore, there is accumulating evidence indicating that under physiological conditions, NO targets cysteine thiols by *S*-nitrosylation and that these covalent modifications modulate the function of several proteins [43]. Interestingly, preliminary data suggest that *S*-nitrosylation of a single cysteine within the functional pore of AQP-1 is associated with a significant loss of water permeability [43, 44]. This finding might help understand the mechanisms of the decreased water permeability of the peritoneal membrane despite a normal expression of AQP1 [35, 45].

It is interesting that in an acute peritonitis rat model, ultrafiltration failure is associated with a major, tenfold increase in peritoneal NOS activity due to the up-regulation of both eNOS and inducible NOS (iNOS) [45]. In this model, up-regulated eNOS is paralleled by a significant increase in vascular density, whereas inflammatory derived cytokines activate the transcription of iNOS.

In humans on long-term PD, regulation of NOS also might be involved in the increased EPSA [33]. Peritoneal NOS activity increases fivefold above control levels and is positively correlated with the duration of PD. Increased NOS activity is solely mediated by calcium-dependent eNOS. Its biological relevance is further demonstrated by concomitant, enhanced immunoreactivity for nitrotyrosine, which points to oxidative stress and increased peroxynitrite formation.

Several factors might contribute to the increased expression of eNOS in long-term PD patients. eNOS up-regulation reflects, at least in part, vascular proliferation and thus increased endothelial surface area. In addition, it may be modulated by changes in immune defense systems induced by long-term PD, such as, local cytokine production [46]. Observations that eNOS is up-regulated in the inflammatory peritoneum [18] or upon virus-induced activation of interferon-γ and tumor necrosis factor-α support the latter hypothesis [47].

In long-term PD patients, advanced glycation and advanced lipoxidation of peritoneal proteins becomes a striking feature [28, 29]. Prior to the onset of PD, in contrast with uremic rats, the AGEs and advanced lipoxidation end products (ALEs) in humans are only faintly detectable. They increase subsequently in parallel with PD duration. Of interest, AGEs colocalize with VEGF along the endothelium lining peritoneal blood vessels [33, 48], and several studies have hypothesized that AGE modification of the peritoneum plays a critical role in its functional alteration [27–29, 33, 49].

PERITONEAL CARBONYL STRESS: THE CAUSE OF AGE MODIFICATION OF THE PERITONEAL MEMBRANE

Advanced glycation of proteins is induced by reactive carbonyl compounds (RCOs) [50]. Several RCOs leading to the formation of AGE epitopes such as pentosidine [51] or CML [52] have been identified, for example, glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone

(3-DG), glycolaldehyde, and arabinose [53–56]. While many others are present and can be estimated by the 2,4-dinitrophenylhydrazine (DNPH) method, their identity remains elusive [29]. In PD, RCOs precursors of AGEs may originate from two sources: the peritoneal dialysate and the uremic circulation [29].

RCOs originating from glucose containing PD fluid

Heat sterilization of PD fluids degrades glucose into products including RCOs, such as GO, MGO, and 3-DG. Research on the role of AGEs in the peritoneum has focused mostly on these RCO precursors [57–59].

RCOs originating from uremic circulation

Plasma RCO precursors of AGEs also have to be taken into consideration as they invade the peritoneal cavity during the dwell time [29]. RCOs, derived not only from carbohydrates but also from lipids, accumulate in uremic plasma ("carbonyl stress") and contribute to the production of AGEs as well as of ALEs in the body [50]. Their accumulation in uremic plasma has been demonstrated, for example, in in vitro incubation experiments during which pentosidine, an AGE moiety, increases over time [60]. Addition of inhibitors of the carbonyl amine reaction, such as aminoguanidine [61] or ±2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) [62, 63], represses the production of pentosidine, pointing to the RCO nature of the pentosidine precursors. Notably, plasma pentosidine levels are correlated with the level of in vitro pentosidine generated after incubation [60], suggesting that they mirror the level of RCO precursors and therefore might be used as a marker of their accumulation [64].

Several mechanisms might account for RCO accumulation in renal failure [65]. Production of RCOs might be enhanced by the oxidative stress associated with uremia [66]. This hypothesis is supported by the positive correlations demonstrated in uremic serum between the levels of pentosidine and markers of oxidative stress such as advanced oxidation protein products (AOPP) [67], dehydroascorbate [68] and superoxide dismutase [69], as well as by the negative correlation reported between serum pentosidine and glutathione peroxidase levels [69]. Alternatively, the rise in RCOs in uremia could be accounted for by a decreased removal by the failing kidneys [64, 70] and/or through enzymatic pathways of detoxification such as the glyoxalase pathway [54]. In the latter pathway, RCOs such as MGO and GO react reversibly with glutathione and are subsequently detoxified by glyoxalases I and II into D-lactate and glutathione. Decreased levels of glutathione therefore can augment the levels of a wide range of RCOs. Glutathione concentration in red blood cells and the serum activity of glutathione-dependent enzymes are significantly reduced in uremic patients [69, 71, 72]. Other, as yet unexplored enzymatic mechanisms also might contribute to a decreased removal of RCOs and thus to the uremic carbonyl stress.

Peritoneal carbonyl stress

We recently demonstrated that not only PD fluid, but also serum-derived RCOs contribute to the genesis of peritoneal AGEs [29]. During the peritoneal dwell time, the RCO content of commercial heat-sterilized glucose PD fluids, that is, GO, MGO and 3-DG, as well as glucose levels fall markedly. In contrast, during the same time interval, total RCO levels (assessed by the DNPH method) increase progressively in the dialysate toward concentrations similar to those observed in plasma. This pattern is compatible with an outward diffusion of GO, MGO, 3-DG from the peritoneal cavity and an inward diffusion of plasma RCOs within the peritoneal fluid.

Plasma derived RCOs are instrumental in the genesis of AGEs, as the generation of pentosidine and CML after incubation of PD effluent increases progressively with dwell time. Further evidence for their role in the modification of peritoneal membrane proteins is provided by the demonstration that ALEs, such as malondialdehyde (MDA)-lysine and 4-hydroxynonenal (HNE)-protein adduct, appear together with AGEs in the peritoneum of long-term PD patients [29]. As already mentioned, uremic plasma RCOs are derived not only from carbohydrates, but also from lipids. The former produce AGEs and the latter produce ALEs. During their diffusion within the peritoneal cavity, uremic RCOs are thus expected to generate not only AGEs, but also ALEs. The presence of the latter adducts, which cannot proceed from glucose degradation products present in commercial heat-sterilized glucose PD fluids, confirm the role played by circulating RCOs crossing the peritoneal membrane during PD.

Advanced glycation end products and ALEs are only faintly present in the peritoneum of uremic patients prior to the onset of PD [28, 29]. Their marked rise after the onset of PD suggests that their formation is enhanced by the augmented mass transfer of plasma RCOs across the peritoneal membrane into the peritoneal cavity, likely as a result of the dialysis procedure itself.

The major RCOs that diffuse from uremic circulation into the peritoneal cavity have yet to be identified. They are not GO, MGO, or 3-DG [29].

Pathological significance of carbonyl stress

The peritoneal cavity of PD patients is thus in a state of severe overload of RCOs derived both from uremic circulation and from conventional glucose-based PD fluids. Which role do these RCOs play in the pathogenesis of peritoneal membrane deterioration?

As stated earlier, RCOs promote the AGE and ALE modification of proteins. In vitro, AGE and ALE modi-

fied proteins initiate a range of cellular responses [73–78], including stimulation of monocyte chemotaxis and apoptosis, secretion of inflammatory cytokines from macrophages, proliferation of vascular smooth muscle cells, stimulation of platelet aggregation, and of VEGF production from endothelial cells. Independently of their AGE- and ALE-mediated effects, RCOs also interfere with various cellular functions and induce both structural and functional alterations of proteins. For example, exposure in vitro of cultured mesothelial and endothelial cells to MGO increases mRNA and protein synthesis of VEGF [48]. Repeated intraperitoneal loads of MGO, given to rats, also increase the peritoneal membrane expression of VEGF in vivo [48]. Noteworthy in this context is the demonstration that, both in long-term PD patients and in the chronic uremic rat model [33, 48], an increasing staining for AGEs, CML and pentosidine is detected in peritoneal arterial walls together with an augmented VEGF and FGF2 expression.

There are two major pathways (direct and indirect) through which carbonyl stress is sensed by cells and triggers a cascade of intracellular signal transduction. In the indirect pathway, the RCOs first interact with proteins or lipids in the physiological environment surrounding the cells, which then undergo nonenzymatic glycation and lipoxidation resulting in the production of AGEs and ALEs. They bind with the receptor(s) on cell surfaces, such as RAGE, thereby initiating intracellular signal transduction [79]. By contrast, the direct pathway works before generation of AGEs and ALEs. The RCOs directly attack target molecules on cell surfaces or inside the cells, which initiate the subsequent signal transduction [80–83]. For example, GO and MGO possess two reactive carbonyl groups to make protein aggregates by cross-linking, which may amplify the signals for tyrosine phosphorylation of cellular proteins [80]. The binding of 4-hydroxynonenal (HNE), a major RCO generated during membrane lipid peroxidation, with epidermal growth factor receptor (EGFR) induces its clustering on the cell surface, thereby activating the mitogen-activated protein (MAP) family kinases [81].

HYPOTHESIS

These new data cast some light on putative mechanisms of peritoneal membrane deterioration in long-term PD patients. They include neoangiogenesis, enhanced growth factor expression, elevated RCO content in the peritoneal cavity, advanced glycation and lipoxidation of peritoneal membrane proteins, and up-regulation of NOS expression with an increased bioactive NO release. In this section, we provide a hypothetical framework to integrate them with the intent to provide clues for future investigations and, possibly, for innovative therapeutic strategies (Fig. 1). Although each step has been documented in vitro, the entire hypothesis remains to be validated in vivo, that is, by testing its various therapeutic implications.

Chronic uremia *per se* modifies the peritoneal membrane and increases the EPSA. Biochemical alterations in the peritoneum inherent to uremia, at least in part, might be accounted for by severe RCO overload originating both from uremic circulation and PD fluid ("peritoneal carbonyl stress"). Plasma RCOs derived from both carbohydrates and lipids accumulate in the uremic circulation and slowly diffuse into the peritoneal cavity. They initiate a faintly detectable AGE as well as ALE protein modifications. During PD, RCOs resulting from heat-sterilization of glucose PD fluid enter the peritoneum, and are complemented by an increased mass transfer of serum RCOs due to the dialysis procedure itself.

The peritoneal carbonyl stress accelerates the AGE and ALE modification of proteins. AGEs as well as their RCO precursors are known to initiate a number of cellular responses. We hypothesize that they stimulate cytokine and growth factors (including VEGF) production and regulate NOS expression in peritoneal cells. The combination of VEGF and NO stimulates angiogenesis, increases permeability, and vasodilates peritoneal capillaries. These combined modifications increase EPSA, which results in faster than normal dissipation of the osmotic and an eventual loss of ultrafiltration. The up-regulation of FGF2 and transforming growth factor-β (TGF-β) also stimulates interstitial fibrosis of the peritoneum [84].

A closer analysis of the interrelationship between RCOs, AGEs, VEGF, eNOS and NO discloses the complexity of the possible pathogenic pathways leading to angiogenesis in the peritoneum. Accumulation of RCOs and AGEs in the peritoneum activates endothelial cells, which promotes VEGF expression. In turn, VEGF further stimulates endothelial cells with an attendant up-regulation of eNOS and the release of NO. The latter contributes, at least in part, to the angiogenic effect of growth factors such as VEGF. Vascular proliferation and increased endothelial area lead to a further up-regulation of eNOS in the peritoneum, and, in some conditions, to sustained release of NO. The latter has a threefold effect. First, it may act as a negative feedback and down-regulate the expression of VEGF [85]. Second, it might target critical cysteine residues and regulate proteins by *S*-nitrosylation [43, 44]. Third, it might decrease AGE production and quench its consequences [86]. Finally, in case of oxidative stress, NO might combine with superoxide and generate peroxynitrite, a labile, cytotoxic reactive oxidant species [87].

These hypothetical mechanisms open a number of therapeutic approaches that might protect the peritoneal membrane against the consequences of long-term PD.

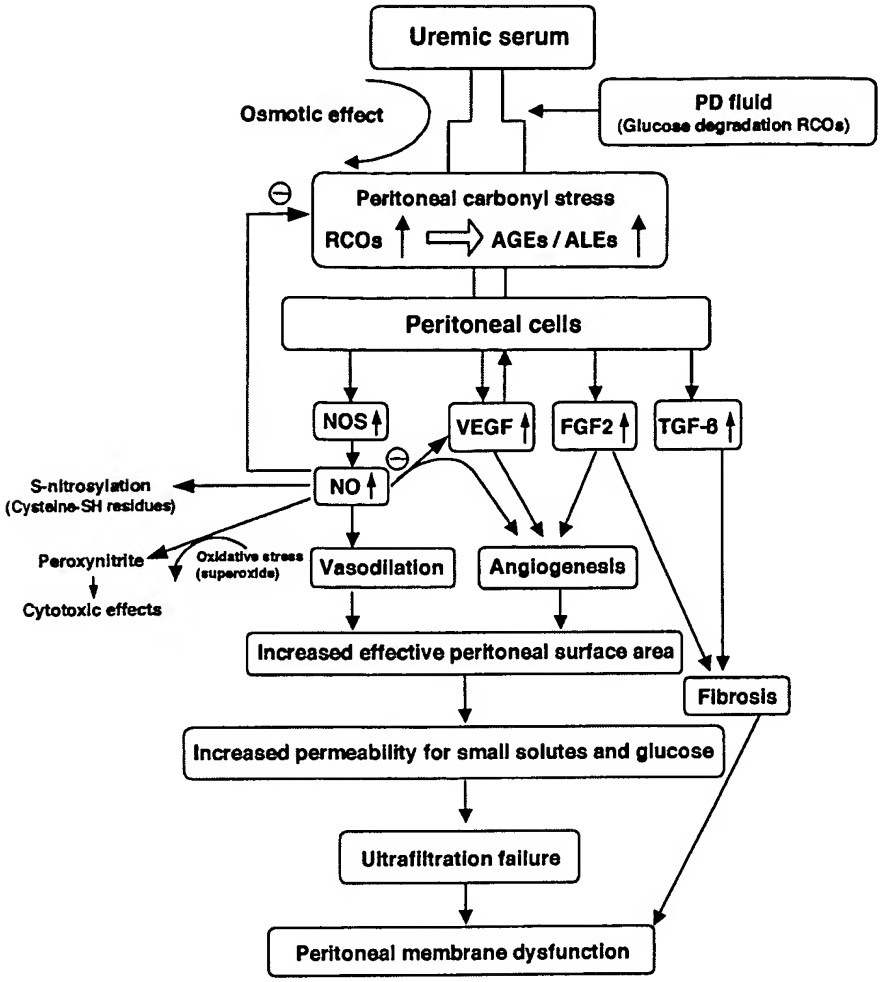

Fig. 1. Hypothesized structural and functional alterations of the peritoneal membrane in long-term peritoneal dialysis (PD). In chronic uremia, plasma reactive carbonyl compounds (RCOs) derived from both carbohydrates and lipids accumulate in the circulation and slowly diffuse into the peritoneal cavity. Biochemical alterations in the peritoneum inherent to uremia are exacerbated by the PD procedure itself. During PD, RCOs resulting from heat-sterilization of glucose PD fluid enter the peritoneum and are complemented by an increased mass transfer of serum RCOs (osmotic effect). The "peritoneal carbonyl stress" accelerates the advanced glycation end product (AGE) and advanced lipoxidation end product (ALE) modification of the peritoneal membrane. AGEs as well as their RCO precursors initiate a number of cellular responses, including cytokine and growth factors [vascular endothelial growth factor (VEGF), basic fibroblast growth factor-2 (FGF2) and transforming growth factor-β (TGF-β)] production, vascular smooth muscle cell proliferation, and specific nitric oxide synthase (NOS) up-regulation. Enhanced VEGF and FGF2 expression, together with an augmented nitric oxide (NO) release, stimulate angiogenesis and vasodilation and increase the permeability of peritoneal capillaries. These combined modifications increase the effective peritoneal surface area (EPSA). The latter augments the permeability for small solutes and glucose, stimulates glucose reabsorption, and results in faster than normal dissipation of the osmotic gradient across the peritoneal membrane with an eventual loss of ultrafiltration. The up-regulation of FGF2 and TGF-β stimulates interstitial fibrosis of the peritoneum.

INNOVATIVE APPROACHES TO MORE EFFECTIVE AND BIOMEDICALLY MORE SUITABLE PERITONEAL DIALYSIS TECHNOLOGIES

Carbonyl stress, increased NO secondary to NOS up-regulation, and up-regulation of VEGF and FGF2 cause some of the morphologic and molecular alterations of the peritoneal membrane. Their manipulation might provide some therapeutic benefits (Fig. 2).

Reduction of the RCO content in peritoneal dialysis fluid or in peritoneal cavity

As previously stated, peritoneal protein modifications are determined not only by RCOs originating from PD fluid, but also by RCOs originating from the uremic circulation. Some strategies have been designed to reduce the RCOs present in PD fluid, and may prove rewarding despite the fact that they do not fully eliminate the peritoneal carbonyl stress.

Glucose-free PD fluids. Major efforts have been undertaken over the last few years to sustain ultrafiltration and to improve nutrition by replacing glucose with icodextrin or amino acids [88–90]. An advantage of these newer solutions accrues from their lower RCO content. GO, MGO, 3-DG as well as total RCO levels are markedly reduced in fresh icodextrin and amino acid PD fluid when compared with heat-sterilized glucose PD fluid [91]. As a consequence, pentosidine and CML generation during incubation is lower in icodextrin and amino acid PD fluid than in conventional glucose PD fluid [91, 92].

The benefits of a lower RCO content of PD fluid are mitigated by the changes in peritoneal fluid RCO content occurring during dwell time [91]. Individual RCOs, such as GO, MGO and 3-DG, fall progressively in icodextrin effluents or remain undetectable in amino acid effluent. By contrast, with dwell time, total RCOs accumulated in uremic serum diffuse within the peritoneal cavity with an attendant increase of the AGE formation potential of PD fluid. While the clinical benefits of these fluids for the preservation of the peritoneal membrane remain to be documented in long-term studies, these findings do

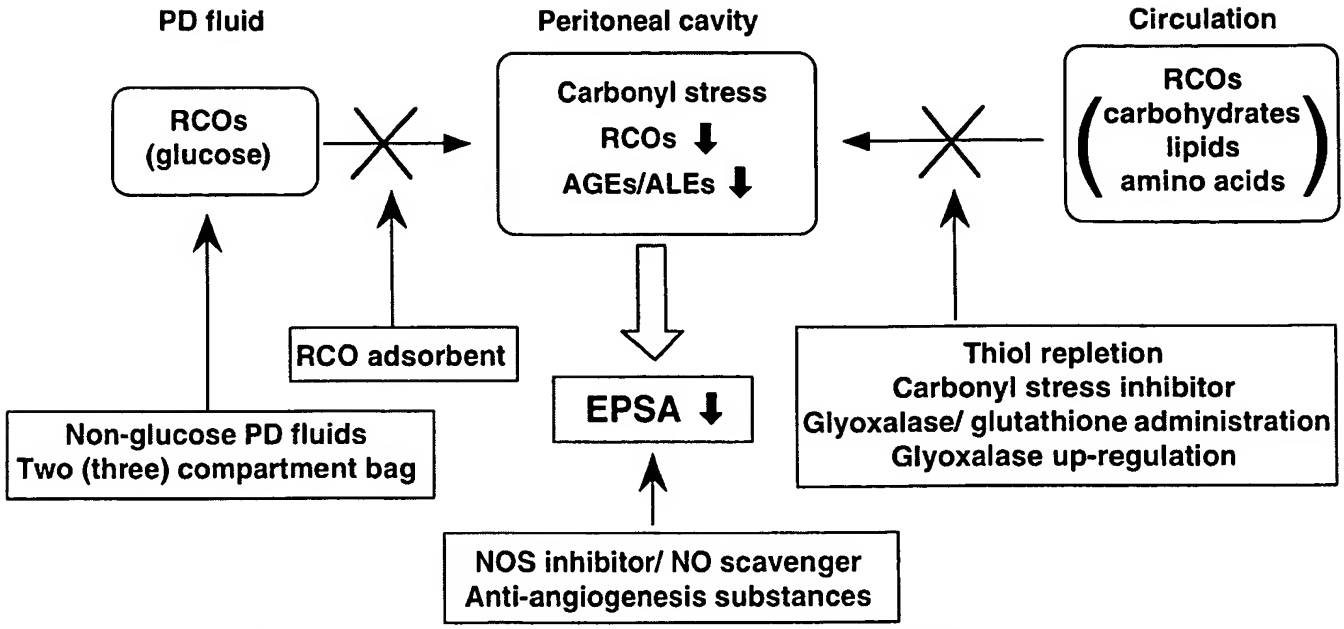

Fig. 2. Innovative approaches to more effective and biomedically more suitable peritoneal dialysis technologies. Peritoneal membrane modifications are determined by RCOs originating from both PD fluid and uremic circulation. PD fluid RCOs are lowered in non-glucose fluids or in multi-compartment bag systems. Peritoneal cavity fluid RCOs metabolism is enhanced by thiol repletion and the addition of glyoxalase I and glutathione. They are trapped by inhibitors of carbonyl amine chemistry or by RCO adsorbents. The increased release of NO, either by constitutive endothelial nitric oxide synthase (eNOS) or by inducible nitric oxide synthase (iNOS) induced by peritoneal inflammation, increases the effective peritoneal surface area (EPSA). These changes are reversible by NOS inhibitors, NO scavengers, and anti-angiogenic substances. The preservation of the peritoneal membrane might require a combination of several therapeutic approaches.

not detract from the clinical merits of these glucose free PD fluids [88]. Icodextrin fluid sustains ultrafiltration profile that is beneficial for long dwells by employing colloidal, rather than crystalline, osmotic pressure, whereas amino acid fluid improves nitrogen balance in patients with malnutrition and avoids the glucose overload, an advantage in diabetic and obese patients.

Multi-compartment bag. An interesting approach to lower the RCO content of glucose-containing PD fluid has been recently provided by a multi-compartment bag system [93, 94]. In this system, glucose is kept at a low pH, separate from the electrolyte buffer (bicarbonate-based) solution at a neutral pH. When both bags are mixed, the final solution has a physiologic concentration of bicarbonate, a reduced concentration of lactate, and a physiologic pH. Its RCO content and AGE generation potential are very low despite conventional heat-sterilization and subsequent storage [95, 96].

The clinical effects of this new fluid have been recently assessed up to two years [97, 98]. Both studies demonstrated elevated effluent levels of cancer antigen (CA) 125 and reduced levels of hyaluronic acid in their overnight effluent, suggesting reduced proinflammatory potential and improved peritoneal membrane integrity. After two years, membrane transport characteristics and ultrafiltration capacity were similar in the double bag and in the control group [97]. Although encouraging, these results should be assessed with caution as a much longer study period may be required to reveal any changes in these parameters.

Inhibitor of carbonyl amine chemistry. An alternative therapeutic strategy might rely on compounds known to inhibit AGE formation. Compounds, such as aminoguanidine, OPB-9195, and probably biguanides [99], contain a hydrazine nitrogen atom that is able to react with carbonyl groups and eventually form hydrazone [63]. Trapping of RCOs by these compounds thus should inhibit the RCO modifications of proteins. Indeed, both aminoguanidine and OPB-9195 inhibit in vitro the formation of AGEs (CML and pentosidine) as well as that of ALEs (MDA-lysine and HNE-protein adduct). AGE generation (pentosidine) from incubated uremic plasma also is inhibited, demonstrating that both compounds trap precursor RCOs of pentosidine accumulating in uremic serum [60].

We have demonstrated that addition of OPB-9195 or aminoguanidine to commercial glucose PD fluids reduced the generation of pentosidine and CML [63], in agreement with a previous study by Lamb et al [100], who reported that aminoguanidine inhibited the fluorescence intensity on albumin incubated in heat-sterilized glucose PD fluid. We further documented a dramatic fall in GO, MGO and 3-DG levels within 24 hours of incubation of PD fluids with OPB-9195 or aminoguanidine [63].

These carbonyl-scavenging agents have further benefits. They block carbonyl stress-mediated intracellular signaling [80, 83]. Oral administration of OPB-9195 to rats, after balloon injury of their carotid arteries, effectively reduces neointima proliferation in arterial walls [62].

Clinical trials of aminoguanidine as well as of OPB-9195 in diabetic patients have been hampered, however, by their neurotoxicity largely due to the trapping of pyridoxal [101]. Less toxic and more specific carbonyl stress inhibitors need to be developed.

Glyoxalase detoxification. Reactive carbonyl compounds partly are detoxified by the glyoxalase pathway. We recently observed a patient in whom a deficiency of glyoxalase I was associated with unusually elevated levels of AGEs (pentosidine and CML) and their precursors [102]. The causes of this deficiency remain unknown. Nevertheless, the association of very low levels of glyoxalase I with strikingly elevated levels of AGEs and RCO precursors offers new therapeutic insights in that maneuvers augmenting glyoxalase I activity might lower RCO and AGE levels.

Preliminary observations in vitro support this approach. Detoxification of RCOs by glyoxalase I is markedly impaired by a decreased thiol concentration (such as glutathione) [103]. Thiol compound such as glutathione, cysteine or *N*-acetylcysteine added to mixtures of GO, MGO, 3-DG decreases their levels. Added to heat-sterilized glucose PD fluid, they lower AGE generation after incubation. Glutathione is undoubtedly less toxic in vivo than aminoguanidine but, unfortunately, less efficient at least in vitro. Addition of glyoxalase I overcomes this limit. Utilized jointly, both compounds dramatically accelerate and intensify the in vitro lowering of GO and MGO both in RCO mixtures and in glucose-based PD fluid. A similar effect is observed when glyoxalase I is replaced by lysates of glyoxylase I transfected cells or by tissue extracts of human glyoxalase I transgenic mice [103]. These results open the exciting prospect of lowering the peritoneal carbonyl stress in humans by raising glyoxalase I activity in peritoneal cells by genetic engineering (described below) or by the concomitant administration of recombinant glyoxalase I with glutathione.

RCO adsorbent. Compounds with RCO binding properties might be immobilized on beads or in a cartridge in order to adsorb RCOs in conventional heat-sterilized glucose PD. Polystyrene sulfonyl hydrazide beads or diaminoguanidine agarose beads added to heat-sterilized glucose PD fluid decrease the levels of GO and MGO as well as the generation of pentosidine and CML after incubation (Miyata T and Ueda Y, unpublished observation).

Inhibitor of the L-arginine-NO pathway

Modulation of the activity of NO may have considerable therapeutic value. The L-arginine analogs, which interfere competitively with the binding of L-arginine to NOS, are well-characterized NOS inhibitors [104]. Despite their lack of specificity, N^G-monomethyl-L-arginine (L-NMMA) and its prodrug N^G-nitro-L-arginine methyl ester (L-NAME) have proved to be the most useful NOS inhibitors [104]. Newer compounds that are more selective toward iNOS [105] and alternative strategies to modulate the biological activity of NO have been considered, but remain to be characterized [106, 107].

Only a few studies have investigated the potential of NOS inhibitors on peritoneal membrane characteristics. Addition of L-NMMA to PD fluid does not alter peritoneal permeability in a chronic PD model in rabbits [108]; however, in different rat models of peritonitis, the addition of L-NAME to PD fluid increases net ultrafiltration with a dose-dependent effect on the permeability for small solutes and proteins [38, 109]. The usefulness of NOS inhibition in long-term PD remains to be defined.

Manipulation of an ubiquitous mediator such as NO raises a number of potential problems and may actually have a double-edged sword effect. Current efforts aim towards improving the selectivity of NOS inhibitors, taking into account differences in expression levels, cofactor utilization and location [104, 105].

Inhibitor of angiogenesis

Based on recent insights [110], a spectrum of strategies for the modulation of angiogenesis has emerged, with the most successful approach to date being the use of agents that inhibit endothelial cell growth [111]. Interference with factors such as VEGF and FGF2 and their receptors may provide another approach. A third approach is to interfere with endothelial cell adhesion and migration [111].

Although more than 30 anti-angiogenesis compounds have been tested in human clinical anti-cancer trials, trials for noncancerous diseases are scarce and limited in scope [111]. No studies have been undertaken in long-term PD, probably because appropriate animal models are lacking [112]. It must be stressed that trials using anti-angiogenic substances require specific approaches to overcome investigator bias [113], and that little information on safety and long-term side-effects of this type of therapy is available.

Gene therapy

Some of functional and structural changes associated with long-term PD might be targeted by gene therapy [114]. Ex vivo gene therapy involves harvesting peritoneum samples to isolate mesothelial cells that will be genetically modified before re-implantation into the peritoneal cavity [115]. The feasibility of this procedure has been demonstrated in animal models by the production of anti-inflammatory and anti-oxidant proteins from genetically modified cells following experimental denuda-

tion of the membrane or chronic exposure to dialysis solutions [115]. While reimplantation of autologous cells in PD patients has been accomplished, conditions for reseeding the peritoneal membrane on an acute or chronic basis still need to be defined. In vivo gene transfer is based on direct gene delivery and in situ genetic modification. The adenovirus system appears to be the most efficient for delivering genes to a significant percentage of mesothelial cells [115]. However, its use in vivo may be limited by local changes resulting from inflammatory and immune responses.

Future prospects of gene therapy in the peritoneum include using either non-viral systems or viruses with low potential for immunogenicity, increasing gene transfer efficiency, and regulating transgene expression through use of mesothelial cell-specific promoters [116].

CONCLUSIONS

Peritoneal dialysis has, among many other factors, the merit of having modified our concept of the peritoneal membrane. Known as a mere envelope of abdominal organs a few decades ago, the peritoneal membrane is now a topic of active research that has already underscored its complex, very active nature.

Evidence is either obtained directly from peritoneal cells and tissue or extrapolated from other cell types to their peritoneal counterparts, and includes information on the composition of peritoneal cavity fluids and their dependence on the uremic environment.

This review focuses on reactive carbonyls and their association with a number of molecular changes observed in peritoneal tissues and with several alterations of peritoneal membrane function. This hypothetical approach will require further testing. Taking into account the complexity of the various systems involved in the maintenance of the peritoneal membrane, it is likely that many of today's assumptions or simplifications will have to be revised and that the present hypothesis will require substantial amendments.

Whatever the fate of the proposed hypothesis, the insights gained on the peritoneal membrane offer a new paradigm to assess the effect of uremic toxins on serosal membranes.

Finally, the progress accomplished in the dissection of the molecular events leading to peritoneal membrane failure open new avenues towards the development of a safe, more biocompatible peritoneal dialysis.

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APPENDIX

Abbreviations used in this paper are: AGEs, advanced glycation end products; ALEs, advanced lipoxidation end products; AQP-1, aquaporin-1; CML, N^{ε}-carboxymethyllysine; 3-DG, 3-deoxyglucosone; EPSA, effective peritoneal surface area; FGF2, basic fibroblast growth factor; GO, glyoxal; HNE, 4-hydroxynonenal; MDA, malondialdehyde; MGO, methylglyoxal; NO, nitric oxide; NOS, nitric oxide synthase; PD, peritoneal dialysis; RCO, reactive carbonyl compound; VEGF, vascular endothelial growth factor.

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The use of affinity adsorbents in expanded bed adsorption

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The potential for the use of affinity ligands in expanded bed adsorption (EBA) procedures is reviewed. The use of affinity ligands in EBA may improve its use in direct recovery operations, as the enhanced selectivity of the adsorbent permits selective capture of the target from complex feedstocks and high degrees of purification. The properties of ligands suitable for use in EBA processes are identified and illustrated with examples. In addition to its use in the recovery of soluble products, such as proteins and nucleic acids, from particulate feedstocks, EBA can also be used to recover particulate entities, such as cells and packaged DNA (viruses and phages), from feedstocks. Affinity ligands coupled to appropriate chosen support materials will be required for such processes in order to achieve the necessary selectivity for the required particulate entity. The latter point is illustrated by the use of proteinaceous ligands immobilized to perfluorocarbon emulsions to achieve separations of microbial cells. © **1998 John Wiley & Sons, Ltd.**

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Introduction

Expanded bed adsorption (EBA) is an increasingly popular technique for the direct recovery of bioproducts from crude, particulate-containing feedstocks such as fermentation broths, cell disruptates, blood and milk (Chase, 1994; Hjorth, 1997). When liquid is pumped upwards through a bed of adsorbent beads which is not constrained by the presence of an upper flow adapter, the bed can expand and spaces open up between the adsorbent beads. Provided that the physical properties of the beads are significantly different from those of the particulates in the feedstock, the particulates can pass freely through these voids in the bed without becoming trapped. This eliminates the need for pre-clarification of crude feedstocks before application to packed beds using centrifugal or filtration techniques and also permits the recovery of particulate bioproducts using column purification techniques. The features of expanded bed adsorption are very similar to those pertaining to packed bed separation, although the former can operate with particulate-containing feedstocks. Expanded bed adsorption can thus be usefully thought of as being a quasi-packed bed procedure and the equipment required and the operating protocols are very similar to those in widespread use in packed bed processes (Chase, 1994).

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Expanded Bed Adsorption for the Isolation of Soluble Bioproducts

The availability of columns and adsorbents specifically designed for expanded adsorption has enabled the feasibility of this technique to be demonstrated in a wide range of systems (Frej *et al.*, 1994, 1997; Hansson *et al.*, 1994; Thommes *et al.*, 1995a,b; Johansson *et al.*, 1996; Noppe *et al.*, 1996; Pessoa *et al.*, 1996; Hjorth, 1997; Maurizi *et al.*, 1997). Adsorbents specially developed for use in EBA can be derivatized with a wide variety of ligands for use in procedures very similar to those widely used in packed bed chromatography. Many reported separations have involved the use of ion exchange adsorbents and in many cases it has been possible to obtain high yields together with effective removal of particulates (Frej *et al.*, 1994; Hansson *et al.*, 1994; Pessoa *et al.*, 1996; Hjorth, 1997). In much of this work the major aim was capture, concentration and clarification of the target rather than to also simultaneously seek high degrees of purification. However, in order to capture selectively the target biomolecule from a crude feedstock containing a wide variety of contaminants, it may be necessary to use affinity ligands. Examples of ligands that have been used in expanded bed separations include biomimetic dyes (Chase and Draeger, 1992; Chang *et al.*, 1995; Garg *et al.*, 1996), protein A (Thommes *et al.*, 1996), heparin (Bjorklund and Hearn, 1996) and metal ions (Jiang and Hearn, 1996). Use of affinity ligands has enabled proteins to be directly purified from crude feedstocks, achieving purification factors of over 100 in high yield. In a comparison of adsorbents for use in expanded bed procedures for the purification of glucose-6-phosphate dehydrogenase from non-clarified cell disruptates of *S.*

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cerevisiae, Chang and Chase 1996a,b) were only able to achieve a purification factor of 12-fold with the ion exchanger STREAMLINE DEAE, whereas use of the biomimetic affinity ligand Procion Red H-E7B covalently attached to the STREAMLINE base matrix resulted in a purification factor of 103 (Chang *et al.*, 1995). In both cases the EBA procedure led to greater than 98% recovery of the target enzyme in a single-step purification procedure that resulted in essentially complete removal of particulates from the product fractions. Use of the same red dye attached to poly(vinyl alcohol)-coated perfluorocarbon particles for the same separation resulted in a purification factor of 172-fold (McCreath *et al.*, 1995). Procion Yellow H-E3G attached to the same perfluorocarbon support was used for the purification of malate dehydrogenase from the same source and a purification factor of 113 was achieved (McCreath *et al.*, 1995).

Immobilized metal ion affinity chromatography (IMAC) may also have applications in the direct recovery of proteins by expanded bed adsorption (Jiang and Hearn, 1996). In packed bed procedures, IMAC has found uses in purification containing $(\text{-His-})_x$ sequences either as a result of the natural amino acid composition of the protein or as a result of the incorporation of histidine tags onto the primary protein structure (Arnold, 1991; Porath, 1992; Brena *et al.*, 1994). Work recently conducted in our laboratory (R. Clemmitt, personal communication) suggests that nickel-loaded STREAMLINE® chelating can be used for the direct recovery of β-galactosidase from unclarified cell disruptates of *E. coli*. The characteristics of the separation (yield and purity) were very similar regardless of whether the adsorbent was used in an expanded bed with the unclarified disruptate or whether the adsorbent was in a packed bed with disruptate that had been clarified by high-speed centrifugation. The addition of the deoxyribonuclease Benzonase® to the disruptate was found to improve the characteristics of the flow of disruptate through the expanded bed and resulted in higher degrees of product capture during the adsorption stage.

Protein A (a cell wall component of *S. aureus*, or recombinant derivatives thereof) is a more complicated affinity ligand that has found application in many procedures for the direct recovery of immunoglobulins from feedstocks such as hybridoma cell cultures (Kennedy, 1996; Thommes *et al.*, 1996; Hjorth, 1997). Although this ligand is proteinaceous, it is remarkably robust and withstands a wide variety of clean-in-place procedures. The use of protein A coupled to an appropriate support matrix such as STREAMLINE may allow smaller beds of adsorbent to achieve higher degrees of product purification when compared with alternative expanded bed processes employing ion exchange adsorbents. In addition, adsorption of immunoglobulin to immobilized protein A can still occur in feedstocks whose conductivity is too great for high degrees of adsorption to ion exchange adsorbents.

Affinity Ligands in Expanded Bed Adsorption

It is important to identify the situations where it is beneficial to use affinity ligands in EBA procedures. Affinity ligands are indicated when insufficient selectivity is achieved with other less selective ligands. The consequence of insufficient selectivity is that many components present in the feedstock become adsorbed to the adsorbent, thus rapidly depleting its overall adsorption capacity. This in turn results in the adsorbent being able to demonstrate only a much reduced adsorption capacity for the target product and necessitates the use of large beds to capture the product from the feedstock. Large beds are undesirable not only as a result of their higher intrinsic costs but also because of the increased volumes of liquids that will be used in washing, elution and clean-in-place procedures. In addition to these detrimental effects on the capture of the target, the fact that the adsorbent has co-adsorbed many other components, it will be inevitable that a lower degree of purification will be achieved during subsequent elution procedures. This is because, unless complicated elution strategies are pursued, other co-adsorbed compounds will be eluted simultaneously. The use of an appropriately chosen affinity ligand should result in adsorption being limited to the target and perhaps a few related compounds containing a similar recognition site. The number of compounds that become adsorbed depends on the nature of the affinity interaction being exploited in the separation.

However, in common with the use of affinity ligands in conventional chromatographic procedures, consideration has to be given to the stability of the ligand, particularly during sanitization procedures, and to the cost and availability of the ligand. Factors important in the choice of an appropriate affinity ligand for use in direct recovery EBA procedures include the stability of the ligand, particularly with respect to its use with very crude feedstocks, and the necessity to subject the adsorbent (with its attached ligands) to harsh clean-in-place procedures. The costs of the ligand have to be considered too, firstly in relationship to development costs associated with finding, selecting and synthesizing an appropriate ligand, but also in relationship to the cost of the ligand when use on the large scale is contemplated. In addition, the use of an affinity ligand in a purification procedure may incur additional quality assurance issues arising from any intrinsic toxicity of the molecule or problems arising from its synthetic origin. The latter is particularly important if a ligand is chosen which has to be synthesized in, and subsequently isolated from, a natural source. Some of the ligands currently emerging from combinatorial approaches to ligand development may be particularly suited to use in expanded bed processes, as these ligands may be robust, cheap and pose fewer regulatory problems (Clackson and Wells, 1994; Krook *et al.*, 1994, 1995; Winter *et al.*, 1994; Caflisch, 1996; Fassina *et al.*, 1996).

Care has also to be taken to ensure that the combination of ligand and base matrix will show a high dynamic adsorption capacity for the target under the conditions of EBA operation. Although the equilibrium adsorption properties are important in this respect, the dynamic adsorption capacity will depend critically on mass transfer limitations in the system. Unlike a packed bed process, which can be operated over a very wide range of flow rates, there is a much smaller operating window of flow rates that can be used in EBA procedures (Hjorth *et al.*, 1995; Thommes *et*

al., 1995a,b; Chang and Chase, 1996a,b). This restriction results from the need to operate the expanded bed at an extent of bed expansion that is typically between two and five times the settled height of the adsorbent. Hence it is always necessary to ensure that the feedstock is allowed adequate residence time in the expanded bed to guarantee high extents of capture of the target. The choice of an appropriate immobilization matrix is especially important in this regard.

It would be wrong to assume that it is beneficial and sensible to use affinity ligands in the direct recovery of all biological products. Indeed, considerations of cost and relative molecular complexity restrict their use to situations where there is a demonstrable benefit; the attractions of simple, less selective ligands dictate their selection wherever possible. There are often circumstances whereby the fundamental biological details of the synthetic system result in no need for the use of affinity ligands in order to achieve high adsorption capacities for the target. Such a situation may occur, for example, during the purification of proteins from systems capable of product over-expression. Commercially viable products are often expressed in biosynthetic systems such that the product constitutes the majority of protein present. This is often the result of directed synthesis of the product towards a location or compartment where it will be a major component relative to other cellular constituents therein. Expression of product into the periplasm and total excretion from the cell to create an exocellular product are examples. Under these circumstances, less selective adsorbents (e.g. ion exchange, hydrophobic interaction) show satisfactorily high dynamic capacities for the product (Frej *et al.*, 1994; Hansson *et al.*, 1994; Thommes *et al.*, 1995a,b; Johansson *et al.*, 1996; Pessoa *et al.*, 1996). In addition to the benefits of increased bed capacity for the product, there is less necessity to employ affinity ligands to achieve purification, as the product is already substantially pure. There is therefore less need to contemplate the use of affinity ligands under such circumstances.

EBA Separations of Nucleic Acids

The EBA technique is also well suited for the purification of soluble bioproducts other than proteins. Advances in gene therapy already require that separations can be made between plasmid DNA, chromosomal DNA and various classes of RNA, and the direct isolation of plasmid DNA from *E. coli* cell lysates using expanded beds of ion exchange adsorbents has been described. Expanded bed techniques were chosen to avoid the need to prepare a clarified feedstock (Hitchcock *et al.*, 1996). There is substantial potential for the development of new affinity ligands to achieve separation of nucleic acids. Separations that simply isolate different classes of nucleic acid can often be achieved by exploiting differences in nucleic acid size and configuration. However, it can be anticipated that such separations may have to be upgraded in order to achieve resolution between different sequences of the same class of nucleic acid. Future regulatory requirements may require the development of novel adsorbents able to achieve such resolution, and new approaches to the development of novel affinity ligands are strongly implicated for these applications (Krook *et al.*, 1994).

Use of Affinity Adsorbents in EBA Procedures for the Separation of Particulate Entities

Although expanded bed adsorption has been mainly used to date in the recovery of soluble (macro)molecules from particulate-containing feedstocks, it is also suited to the recovery of particulate entities such as cells, viruses, virus-like particles, packaged DNA and other particulate products. Particulate entities cannot normally be isolated using conventional packed bed procedures, as a result of the tendency of these particulates to become entrapped within the static voids of the bed with consequent loss of yield. The extent of the loss of particulates depends on their relative size compared with the beads of adsorbent in the packed bed. However, the higher voidages present in expanded beds allow efficient contact of particulate entities with the external surface of the adsorbent beads in the bed, thus permitting adsorption and subsequent elution without entrapment. Washing and elution can also be carried out in the expanded mode, thus enabling the release and collection of the particulate entities during the elution phase of the process.

Affinity ligands may be well suited to the recovery and separation of particulates, especially if the chosen ligand's selectivity can result in distinction between different types of particulates. Such differential selectivity requires exploitation of the biochemical nature of surface components and is less likely to be achievable with less selective ligands, as evidenced by the fact that cells and cell debris do not often overwhelm the capacity of ion exchange adsorbents (Draeger and Chase, 1991; Pessoa *et al.*, 1996). In some cases reported to date, the required selectivity to separate different cell types has had to be achieved by using macromolecular ligands, e.g. the use of antibodies for selecting specific cell types (Widjojoatmodjo *et al.*, 1993; Wipat *et al.*, 1994; Fluit *et al.*, 1995; Kvalheim *et al.*, 1996). However, as has been described above, there is gathering evidence that such ligands, which are unattractive for use in validated bioprocesses, could be replaced with ligands discovered and identified by combinatorial approaches.

The much larger overall size of particulate entities suggests that porous adsorbents of the type conventionally used to purify soluble (macro)molecules are less likely to be well suited for the adsorption of particulates. These entities are unlikely to be able to penetrate porous adsorbents, and the use of ultraporous materials may be fraught by slow rates of diffusive mass transfer or the possibility of pore blockage. The latter may also occur if there is, to some extent, convective flow through the adsorbent bead. For these reasons it may be prudent to select adsorbents for use in such applications that are either non-porous or have only a limited porosity, penetrable by large entities to access adsorption sites located at short diffusional distances from the bulk flow. Solid perfluorocarbons coated with poly(vinyl alcohol) (PVA), which have already been described above for the purification of soluble biomolecules, may be

appropriate for such purposes. In addition, droplets of liquid perfluorocarbons (e.g. perfluorodecalin) stabilized by the adsorption of PVA on their surface (i.e. their interface with the aqueous phase), firmly held in place by chemical crosslinking, may be particularly suited for the adsorption of cells. The elastically deformable nature of these droplets may be important when components on the cell surface form reversible chemical complexes with the surface of the droplet.

The use of non-porous particles normally necessitates the use of particles with small diameters in order to ensure high capacities per unit volume of bed. If small particles are used in packed bed processes, the major consequence is the resultant higher pressure drop across the bed. On the other hand, if used in an expanded bed, the smaller terminal velocities of these small particles dictate operation at inconveniently slow superficial flow velocities. One solution to the latter problem is the use of small particles with high bulk densities to increase particle terminal velocities. There is substantial scope for the development of novel matrices which exhibit the desired physical properties for such separations.

Affinity Separation of Cells

McCreath and Chase (1996a,b) have reported the use of PVA-coated perfluorodecalin-in-water emulsions with an average droplet diameter of 10 μm for the affinity separation of cells in batch procedures. These procedures have involved covalent derivatization of the emulsion surface with affinity ligands, namely concanavalin A for the adsorption of *Saccharomyces cerevisiae* and human IgG for the adsorption of *Staphylococcus aureus*. Studies of the covalent immobilization of immunoglobulin G showed that up to 2.5 mg of protein could be immobilized per settled ml of the emulsion. These affinity emulsions could then be used to adsorb up to 4.8×10^{10} cells of *S. aureus* per ml of settled emulsion. Equivalent experiments conducted with concanavalin A for the adsorption of *S. cerevisiae* showed protein immobilization levels of up to 2.1 mg of concanavalin A per ml and adsorption capacities of 6×10^9 cells per ml. As 1 ml of settled emulsion contains 1.7×10^9 droplets, these cell capacities correspond to approximately 30 and four cells per droplet for *S. aureus* and *S. cerevisiae* respectively. The kinetics of cell adsorption was also found to be rapid, with 50% uptake occurring within 30 s in systems with excess capacity for adsorption of cells. The IgG emulsion showed a very selective affinity for *S. aureus* even in the presence of a 780-fold excess of *S. cerevisiae* cells, with no evidence of any *S. cerevisiae* becoming bound to the emulsion. It was shown that these PVA-coated emulsions demonstrated no non-specific adsorption of cells and it was not necessary to include detergents or other blocking agents to prevent non-specific adsorption.

These results begin to pave the way towards carrying out cell separations in expanded beds as an alternative to immunomagnetic cell separations (Ugelstad *et al.*, 1992, 1993; Ellingsen *et al.*, 1993; Widjojoatmodjo *et al.*, 1993). Whereas the latter are already in use, it is anticipated that cell separations carried out in expanded beds may be performed with simple equipment in a cost-effective manner.

Conclusions

It has been demonstrated that affinity ligands have considerable potential for use in EBA procedures. These improvements arise from increased adsorbent selectivity, which results in higher capacities of adsorbent for the target and higher degrees of purification. It is envisaged that EBA techniques using affinity ligands will also be used to separate cell types and other particulate entities. There is, however, an overwhelming need for novel affinity ligands for use in applications such as nucleic acid purification and cell separation, and use for these purposes must constitute an important goal for combinatorial ligand synthesis

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Removal of advanced glycation end products in clinical renal failure by peritoneal dialysis and haemodialysis

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Abstract

AGEs (advanced glycation end products) accumulate markedly in the plasma of human subjects with renal failure. We investigated the efficiency of removal of AGEs from the circulation by PD (peritoneal dialysis) and HD (haemodialysis) therapy. Free AGEs were measured by LC-MS/MS in blood plasma before dialysis, in dialysis fluid effusate after a 2–12 h dwell time in the peritoneal cavity of PD subjects, and in the HD dialysate before and after HD therapy. In clinical uraemia, the concentrations of free AGEs in blood plasma were increased up to 50-fold. For example, levels of MG-H1 (methylglyoxal-derived hydroimidazolone) were: normal controls, 110 ± 46 nM; PD subjects, 1876 ± 676 ($P < 0.01$); HD subjects, 5496 ± 1138 nM ($P < 0.001$). In PD subjects, the AGE concentration in the effusate increased with increasing dwell time, reaching a maximum at a concentration higher than that in plasma for some AGEs at 4–12 h. This may reflect AGE formation in the peritoneal cavity. In HD, AGE concentrations in HD fluid were decreased markedly from the start to the end of a dialysis session, except that levels of the methylglyoxal-derived AGEs N^{ε}-(1-carboxyethyl)lysine and MG-H1, and of pentosidine, remained 5-fold higher than control levels. Inadequate clearance of free AGEs may be linked to the increased risk of cardiovascular disease in patients with renal failure.

Introduction

A key aspect of the hypothesis that advanced glycation is insidious to health is the need for degradation of AGE (advanced glycation end product)-modified proteins and renal elimination of AGEs. AGEs were found to accumulate in plasma of diabetic and non-diabetic patients with ESRD (end-stage renal disease) [1]. Kidney transplantation decreased plasma AGE levels [2]. AGEs have been detected as unidentified antigens in AGE-specific ELISAs [1,3] and using chromatographic techniques for detection of CML (N^{ε}-carboxymethyl-lysine) and the cross-links GOLD (glyoxal-derived lysine dimer) and MOLD (methylglyoxal-derived lysine dimer) [4,5]. Recently, however, several further complicating factors have emerged. Serum proteins of non-diabetic patients with uraemia on dialysis had abnormally high glycation – particularly in subjects with continuous ambulatory PD (peritoneal dialysis). Dialysis has, in some instances, increased the serum AGE concentration in patients with uraemia [6,7].

Abnormalities of glycation and clearance of AGEs in uraemia may be complicated by the effect of oxidative stress induced by polymorphonuclear leucocyte activation by uraemic toxins and by interaction with the dialysis membrane in HD (haemodialysis) [8], and by the introduction of high concentrations of glucose in dialysis fluids in PD and continuous ambulatory PD (74–214 mM). Oxidative stress decreases the cellular concentrations of glutathione and NADPH [9]. GSH is the cofactor for the detoxification of glyoxal and methylglyoxal by the glyoxalase system [10]; NADPH is the cofactor for the detoxification of 3-deoxyglucosone by 3-deoxyglucosone reductase [11]. Hence glyoxal, methylglyoxal and 3-deoxyglucosone accumulate in oxidative stress [12] and thereby AGE formation is increased. High concentrations of glucose (and α-oxoaldehydes [13]) in dialysis fluids also increase glycation. More research is therefore required to characterize the efficiency of AGE clearance in renal dialysis protocols. We investigated the levels of AGE residues in proteins and free AGEs in blood plasma of human subjects with ESRD, each receiving one of two types of dialysis therapy, PD or HD, and normal healthy controls (Table 1 and Figure 1). AGEs were determined by a quantitative comprehensive screening assay by LC-MS/MS with internal standardization by stable isotope-substituted standards and calibration by reference

Key words: advanced glycation end product (AGE), haemodialysis, peritoneal dialysis, renal failure, uraemia.

Abbreviations used: AGE, advanced glycation end product; CEL, N^{ε}-(1-carboxyethyl)lysine; CML, N^{ε}-carboxymethyl-lysine, 3DG-H, 3-deoxyglucosone-derived hydroimidazolone {N^{δ}-[5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl]ornithine}; ESRD, end-stage renal disease; G-H1, glyoxal-derived hydroimidazolone [N^{δ}-(5-hydro-4-imidazolon-2-yl)ornithine]; GOLD, glyoxal-derived lysine dimer; HD, haemodialysis; MOLD, methylglyoxal-derived lysine dimer; MG-H1, methylglyoxal-derived hydroimidazolone [N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine]; PD, peritoneal dialysis.

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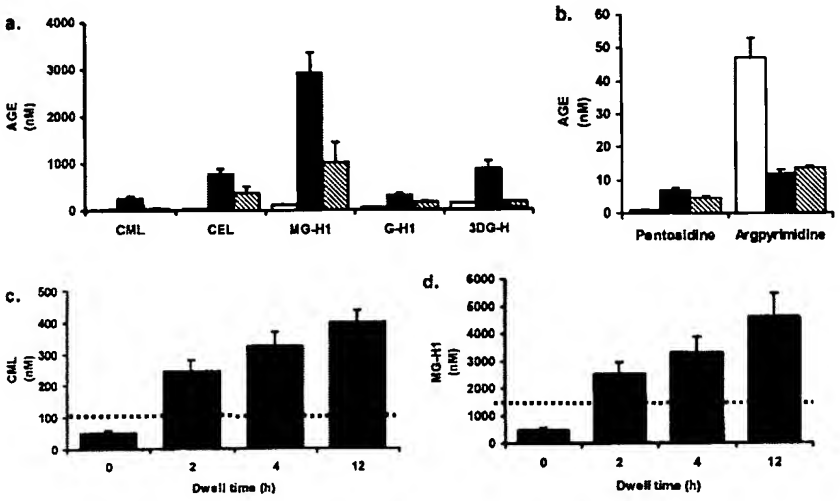
Table 1 | Accumulation of free AGEs in plasma of human subjects with renal failure

Data are means ± S.D. or median (range) for $n = 5$–6. Significance: P values are significance levels with respect to control and PD subjects respectively.

	Plasma concentration (nM)		
AGE	Normal controls	PD patients	HD patients
CML	23 ± 8	108 ± 48 ($P < 0.01$)	200 ± 35 ($P < 0.001$)
CEL	35 ± 14	388 ± 180 ($P < 0.01$)	817 ± 263 ($P < 0.001$)
G-H1	50 ± 17	167 ± 64 ($P < 0.01$)	237 ± 38 ($P < 0.001$)
MG-H1	110 ± 46	1876 ± 676 ($P < 0.01$)	5496 ± 1138 ($P < 0.001$)
3DG-H	147 ± 19	1137 ± 531 ($P < 0.01$)	1541 ± 520 ($P < 0.01$)
MOLD	15 ± 6	13 ± 8	14 ± 5
Argpyrimidine	47 ± 13	71 (3–259)	90 (23–501)
Pentosidine	0.84 ± 0.50	2.35 ± 1.66	4.91 ± 2.76 ($P < 0.01$)

Figure 1 | Free AGEs in dialysate fluid in human subjects receiving HD or PD therapy

(**a**, **b**) Patients on HD therapy. Key (from the left): first series (open bars), plasma of control subjects ($n = 5$; see Table 1); second series (solid bars), haemodialysate at the start of a dialysis session; third series (hatched bars), haemodialysate at the end of a dialysis session ($n = 8$). (**c**, **d**) CML and MG-H1 levels in the dialysate of patients on PD therapy. The broken lines indicate the concentrations of free CML and MG-H1 in the plasma of the same subjects at a dwell time of 2 h. Data are means ± S.E.M.



to calibration curves produced with authentic reference AGEs [14].

Free AGEs in plasma and dialysates in clinical dialysis

The plasma concentration of free CML was increased approx. 4-fold in PD subjects and 8-fold in HD subjects. The concentration of free CEL [N^{ϵ}-(1-carboxyethyl)lysine] was increased more markedly than that of free CML, however, increasing approx. 10-fold in PD subjects and 22-fold in HD subjects. Therefore the concentrations of free CML and CEL were increased markedly in ESRD. Free hydroimidazolone AGEs were also increased markedly and severely in PD and HD subjects. G-H1 [glyoxal-derived hydroimidazolone; N^{δ}-(5-hydro-4-imidazolon-2-yl)ornithine], MG-H1 [methylglyoxal-derived hydroimidazolone; N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine] and 3DG-H {3-deoxyglucosone-derived hydroimidazolone; N^{δ}-[5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl]ornithine} were increased by 2-fold, 16-fold and 7-fold respectively in PD subjects, and by 4-fold, 50-fold and 10-fold respectively in HD subjects. The concentration of free pentosidine was not increased significantly in PD subjects, but was increased 5-fold in HD subjects.

In HD, AGE concentrations in the HD fluid were decreased markedly from the start to the end of a dialysis session, except that levels of the methylglyoxal-derived AGEs

CEL and MG-H1, and of pentosidine, remained 5-fold higher than controls levels. In PD subjects, the AGE concentration in the effusate increased with increasing dwell time, reaching a maximum at a concentration similar to that in blood plasma by 4–12 h. The concentrations in blood plasma of CML and MG-H1 at a 2 h dwell time (Table 1 and broken line in Figures 1c and 1d) were lower than the concentrations in the peritoneal dialysate. This suggests that there is some active accumulation of free AGEs in the peritoneal cavity or that there is increased formation of AGEs in the peritoneal cavity. The latter is possible, since there are high concentrations of glucose and α-oxoaldehydes in the commercial PD fluids used.

AGE accumulation in ESRD was expected, because the normal renal excretion of AGEs was markedly decreased and the concentrations of α-oxoaldehyde precursors of AGEs, i.e. glyoxal, methylglyoxal, 3-deoxyglucosone and ribosone (a possible precursor of pentosidine), were markedly increased [15,16]. The extraordinarily marked accumulation of free AGEs – particularly MG-H1 and 3DG-H – in PD and HD subjects may reflect increased protein glycation by the high concentrations of methylglyoxal and 3-deoxyglucosone in these subjects [15,16]. Free AGEs probably originate mainly from the degradation of protein glycated endogenously and from AGEs absorbed from food. Lack of effective clearance of AGEs leads to their accumulation. The increased endogenous glycation in uraemia arising from increased α-oxoaldehyde levels will exacerbate this effect, particularly when glyoxalase I activity is low – as found for a uraemic subject that had an extraordinarily low glyoxalase I activity [17]. The high risk of cardiovascular complications associated with decreased glyoxalase I activity, despite controlling for other known risk factors in this subject, suggested that α-oxoaldehyde-mediated glycation may be linked to cardiovascular complications in uraemia. Inadequate clearance of free AGEs may also be linked to the increased risk of cardiovascular disease in patients with renal failure. Accumulation of free AGEs in uraemia may be a surrogate indicator of the effectiveness of dialysis therapy and a risk indicator or factor for vascular complications.

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Hyperglycemia, glycoxidation and receptor for advanced glycation endproducts: potential mechanisms underlying diabetic complications, including diabetes-associated periodontitis

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Diabetes mellitus is associated with a wide range of complications that increase morbidity and mortality in affected individuals. These complications ensue from abnormal regulation of glucose metabolism that characterizes diabetes. In the United States alone, it is estimated that diabetes afflicts more than 12 million people (43). In the macrovasculature, accelerated and aggressive atherosclerosis portends the development of premature cardiovascular and cerebrovascular events (35, 46, 84). Microvascular disease may lead to the devastating complications of blindness and renal failure (5, 83). In certain complications, such as impaired wound healing, impotence and neuropathy, dysfunction of vascular, inflammatory and neural components together contribute to progressive impairment of cellular structure and function (3, 20, 54, 85). Furthermore, it is established that the prevalence, severity, and progression of periodontal disease is increased in patients with diabetes (14, 18, 26, 57, 77).

Impaired host resistance likely underlies the altered response to infection that occurs in diabetic subjects. For example, impaired recruitment and phagocytic function of neutrophils has been linked to excessive tissue destruction by pathogenic bacteria (45, 55). In addition, an exaggerated inflammatory response to mediators such as lipopolysaccharide has been suggested to contribute to aggressive tissue damage in diabetic individuals (53, 59). In this context, it has recently been demonstrated that, in diabetes, an upregulated proinflammatory monocyte response results in enhanced production of tumor necrosis factor-α, interleukin 1-β and prostaglandin E_2, findings linked to increased severity of periodontal disease (65, 66). Other studies have linked the increased severity of periodontal disease in diabetic subjects to genetic predisposition, with exaggerated immune responses to bacterial challenge contributing to increased tissue destructive processes (95). Furthermore, impaired generation of collagen, as well as exaggerated collagenase activity, have been implicated in the pathogenesis of diabetic periodontitis (61, 67).

Taken together, these factors strongly suggest that, in diabetes, a number of abnormal host effector mechanisms converge to lead to a range of complications. Delineation of those mechanisms, also important in the pathogenesis of diabetes-associated periodontitis, will be critical in the design of targeted therapy to delay or prevent exaggerated periodontal destruction and tooth loss in diabetic individuals.

Hyperglycemia: direct and indirect sequelae

Diabetes is a group of metabolic disorders. Deficiency of insulin and impaired cellular sensitivity to insulin (insulin resistance) are defined as types 1

and 2 diabetes, respectively. Impaired glucose tolerance, gestational diabetes and drug- or chemical-induced diabetes, for example, are part of the spectrum of the associated disorders. Regardless of etiology, however, common is the presence of elevated serum glucose. Direct and indirect consequences of hyperglycemia have been described that likely play contributory roles in the pathogenesis of the long-term complications that characterize diabetes.

Direct consequences of hyperglycemia

One consequence of elevated levels of glucose is the increased production of sorbitol and fructose by the enzyme aldose reductase. Under homeostatic, normoglycemic conditions, this enzyme, which has a low affinity for glucose, usually processes little substrate. However, in hyperglycemia, markedly increased production of sorbitol ensues. This process has long been hypothesized to be linked to the development of diabetic retinopathy, neuropathy and nephropathy (19, 22, 47). In certain populations, recognized polymorphisms in the aldose reductase gene have been associated with increased complications (29, 39). Active debate remains, however, with respect to therapeutic effectiveness of inhibitors of aldose reductase. It is nevertheless apparent that, at least with currently available inhibitors of aldose reductase, ocular and renal sequelae of diabetes continue to occur.

A so-called downstream effect of elevated levels of blood glucose results from the increased production of diacylglycerol, one consequence of which is activation of protein kinase C, especially the β isoform. Activation of protein kinase C has been hypothesized to be linked to the development of diabetic retinopathy and nephropathy. Indeed, administration of inhibitors of protein kinase C-β is under testing in diabetic patients (2, 34, 40).

Despite evidence linking elevated levels of serum glucose to enhanced complications in diabetes, it is probable that the direct consequences of hyperglycemia do not solely underlie the predisposition to microvascular, macrovascular, inflammatory and neural cell complications. Indeed, the results of the Diabetes Control and Complications Trials Research Group demonstrated diminished complications of the microvasculature with strict control of hyperglycemia in patients with type 1 diabetes (82). However, these complications were not completely eliminated. Furthermore, rigorous control of hyperglycemia did not significantly impact on the development of macrovascular disease. These findings strongly suggest that even intermittent periods of hyperglycemia may result in serious long-term and irreversible sequelae. Such factors, we hypothesize, are an important component in the development of many diabetes-related complications.

Indirect consequences of hyperglycemia

It is well established that exposure of the body's proteins and lipids to reducing sugars leads to the initial formation of reversible products of nonenzymatic glycation and oxidation, the Schiff bases and Amadori products. The best-known of these products is glycosylated hemoglobin A_{1c}, whose measurement is used as a clinical barometer of glucose control over weeks to months (17). After a series of further complex molecular rearrangements, the irreversible advanced glycation end-products (AGEs) are formed (6, 12, 64, 76). While a range of structures in this heterogeneous class of compounds has been described, such as carboxymethyl(lysine), pentosidine, pyralline and methyglyoxal, for example, the structure(s) responsible for cellular perturbation have yet to be fully delineated (33, 52, 68, 92). AGEs form and accumulate in a number of circumstances, such as aging, renal failure and diabetes. Indeed, the presence of AGEs in diabetic plasma and tissues has been linked to the development of diabetic complications (10). Recent observations that polyol metabolites themselves may lead to generation of AGEs (27) suggest that, not unexpectedly, seemingly diverse pathways may converge to form these modified adducts. Interestingly, recent studies suggested that certain AGEs, carboxymethyl(lysine) modifications of proteins, may form as a consequence of activation of the myeloperoxidase-hydrogen peroxide-chloride system, thereby providing a mechanism for direct generation of AGEs in inflammatory milieu, even in euglycemia (4).

Accumulation of AGEs in the tissues may result in significant alteration of normal cellular composition and structure. Cross-linking of long-lived proteins such as collagen, for example, may lead to abnormal barrier function and integrity, as well as the trapping of macromolecules, such as low density lipoproteins. In addition, nonenzymatic glycoxidation of basement membrane-associated structures may prevent their facilitation of cell attachment, and modification of growth factors may suppress mitogenic activity (21, 25).

In addition to apparently receptor-independent mechanisms, AGEs may also interact directly with cell surfaces. While a number of putative cell surface

binding sites for AGEs have been identified (36, 56, 71, 89, 99), the best characterized of these is the receptor for AGE (RAGE), a member of the immunoglobulin superfamily of cell surface molecules. Molecular cloning of RAGE and the putative hydropathy plot revealed that RAGE consists of a 332-amino-acid extracellular region containing one "V"-type immunoglobulin domain, followed by two "C"-type domains. This portion of the molecule is followed by a hydrophobic transmembrane spanning domain, and, lastly, by a highly-charged cytosolic tail of 42 amino acids, which is essential for RAGE-mediated signal transduction (30, 37, 56). Furthermore, recent studies have suggested that physiologically relevant levels of carboxymethyl(lysine) modified proteins may ligate RAGE to alter cellular properties, largely via its "V"-type immunoglobulin domain (37).

The receptor for AGE and target cell dysfunction

The interaction of AGEs with RAGE perturbs specific cellular function

In homeostasis, RAGE is present at low levels in a number of cell types, including endothelial cells, smooth muscle cells, neurons and monocytes (9, 71). However, in perturbed states, such as diabetes, renal failure, Alzheimer's disease, and inflammation, for example, the expression of RAGE on critical target cells is strikingly enhanced (1, 8, 9, 30, 49, 74, 79, 96). Interestingly, RAGE is highly expressed in neurons of the developing central nervous system. In *in vitro* studies the receptor co-localizes with increased levels of the polypeptide amphoterin, with which it interacts to mediate neurite outgrowth (32). Therefore, beyond the binding of RAGE with pathophysiological ligands such as AGEs, its interaction with amphoterin, we speculate, likely offers a clue to its "natural function" (96, 98).

Endothelial cells. RAGE is present at low levels on endothelial cells under normal conditions; upon perturbation, however, the expression of RAGE is increased (1, 8, 9, 74, 79). AGEs bind to cultured endothelial cells with $K_d \approx 50$ nM, in a RAGE-dependent manner. In our studies, we have used a number of different types of AGEs both those prepared *in vitro* and those isolated from human subjects, such as AGE β_2-microglobulin or AGEs that form or deposit on the surface of diabetic red blood cells (90). In all cases, the binding of these AGEs to endothelial cells was blocked in the presence of either anti–RAGE IgG or excess soluble RAGE (sRAGE; the extracellular two-thirds of RAGE that contains the ligand binding site) (69). The interaction of AGEs with endothelial RAGE resulted in the development of a range of perturbations likely linked to the development of vascular lesions in diabetes. For example interaction between AGEs and endothelial RAGE led to increased monolayer permeability (91) and increased production of vascular cell adhesion molecule-1 (70). These processes were blocked by either anti–RAGE IgG or sRAGE. *In vivo*, infusion of AGEs into normal mice resulted in increased production of interleukin 6 (69), another mediator closely associated with the development of cellular perturbation.

Mononuclear phagocytes. Inflammatory cells such as mononuclear phagocytes play a central role in the development of vascular lesions induced, for example, in the presence of highly oxidized lipoproteins. In this context, altered monocyte activity has been linked to the pathogenesis of accelerated atherosclerosis (63). Further, monocyte binding to modified adducts such as AGE β_2-microglobulin (49–51) may be important in the pathogenesis of bone and joint destruction observed in patients with dialysis-related amyloidosis.

Similarly to the situation in cultured endothelial cells, AGEs bind to human peripheral blood-derived mononuclear phagocytes with $K_d \approx 50$ nM. Further, the interaction of AGEs with monocyte RAGE resulted in enhanced chemotaxis of monocytes. Both processes were blocked in the presence of either anti–RAGE $F(ab')_2$ or sRAGE (49, 73). While soluble AGEs resulted in directed chemotaxis of monocytes, when AGEs were immobilized on the surface of upper membranes in a modified chemotaxis chamber, monocytes were retained (haptotaxis). This occurred in a RAGE-dependent manner. When polytetrafluoroethylene tubes were incubated with AGE rat serum albumin and implanted into the backs of rats, a florid attraction and deposition of mononuclear inflammatory cells ensued. Incubation of polytetrafluoroethylene tubes with native rat serum albumin incited minimal inflammatory response, except at the tissue-graft interface (Fig. 1).

In addition to mediating migration of monocytes, interaction between AGEs and monocyte RAGE further results in their activation. For example, incubation of AGE β_2-microglobulin with human monocytes resulted in increased release of tumor necrosis factor-α. Production of this cytokine was blocked in the presence of sRAGE (49). These data

strongly suggested that the interaction of AGEs with monocyte RAGE might have implications for a range of circumstances in which AGEs form and accumulate, such as in diabetes.

Smooth muscle cells. RAGE is present on the surface of vascular smooth muscle cells (9) and mediates interaction with AGEs. The interaction of AGEs with cultured smooth muscle cells resulted in their increased migration and activation (23). In this context, then, increased interaction of AGEs with smooth muscle cell RAGE has important implications for vascular perturbation and injury.

Fibroblasts. Our studies have also indicated that RAGE is present on cultured human fibroblasts (59). Incubation of fibroblasts with AGE β_2-microglobulin resulted in decreased production of type I procollagen messenger RNA in a time- and dose-dependent manner. This did not occur in the presence of anti-RAGE IgG, but was seen when non-immune IgG was added to the cultures (59). Similarly, the synthesis of type I collagen was decreased in the supernatants of AGE β_2-microglobulin-treated fibroblasts in a RAGE-dependent manner as exemplified by experiments with anti–RAGE IgG. These studies suggested that AGE-RAGE interaction may be an important contributory factor in impaired remodeling of connective tissue observed in diabetes.

Taken together, these data indicated that the interaction of AGEs with RAGE on a number of cell types may play an important role in the development of vascular and inflammatory cell dysfunction that underlies the microvascular, macrovascular and inflammatory complications that characterize diabetes.

The interaction of AGEs and RAGE results in induction of enhanced cellular oxidant stress

Our laboratory then started examining the principal mechanisms by which all the above effects ensue. Our data have demonstrated that, in part, it is via the induction of cellular oxidant stress (42, 72, 97). When endothelial cells were cultured with AGE albumin, increased generation of thiobarbituric acid reactive substances (an indirect marker of lipid peroxidation) occurred in a RAGE-dependent manner. Northern blotting of endothelial cells treated with AGE albumin revealed increased messenger RNA for heme oxygenase-1, a marker of enhanced oxidant stress. In addition, electrophoretic mobility shift assay of nuclear extracts prepared from AGE-treated endothelial cells revealed activation of nuclear factor-κB. Both processes were blocked in the presence of RAGE blockade, as well as antioxidants, such as probucol or *N*-acetylcysteine (97). Similar results were obtained in *in vivo* studies. Infusion of AGE albumin into normal mice resulted in a time- and dose-dependent increase in thiobarbituric acid reactive substances in tissues such as brain, lung and gingiva. The increase in thiobarbituric acid reactive substances was again blocked in the presence of RAGE blockade and antioxidants (72, 97). Consistent with these data, infusion of AGEs into normal mice resulted in enhanced activation of nuclear factor-κB. This occurred in a RAGE- and oxidant-dependent manner.

Taken together, these studies indicated that one of the mechanisms by which the interaction of AGEs with RAGE alters cellular phenotype and function is by activation of cell-signaling pathways that eventuate in activation of nuclear factor-κB, an important mediator of the inflammatory response (15).

Does antagonism of AGE-RAGE interaction offer a novel therapeutic approach in diabetes?

The enhanced expression of RAGE in diabetic tissue as well as the pathological sequelae of AGE-RAGE interaction on target cells point to this interaction as a possible novel therapeutic target. Other preventive and therapeutic approaches have included efforts such as strict euglycemic control (82), aldose reductase inhibitors (19, 22, 47) and inhibitors of protein kinase C (2, 34). The indirect effects of hyperglycemia, especially those eventuating in irreversible consequences, however, must be considered important in the design of complementary therapeutic targets in diabetes. In this context, agents such as aminoguanidine (with multiple modes of possible action, including diminished formation of AGEs and possibly antioxidant function) are being tested to prevent diabetic complications (11, 16, 28, 75, 78, 80, 81). In addition, agents designed to cleave preformed AGEs have been suggested (87). Issues of *in vivo* efficacy and safety, however, remain to be established.

We have postulated that sRAGE may represent a novel tool for the therapy of diabetic complications based on initial studies in *in vitro* binding and cell culture assays and in *in vivo* animal models. The binding of radiolabeled AGE β_2-microglobulin to im-

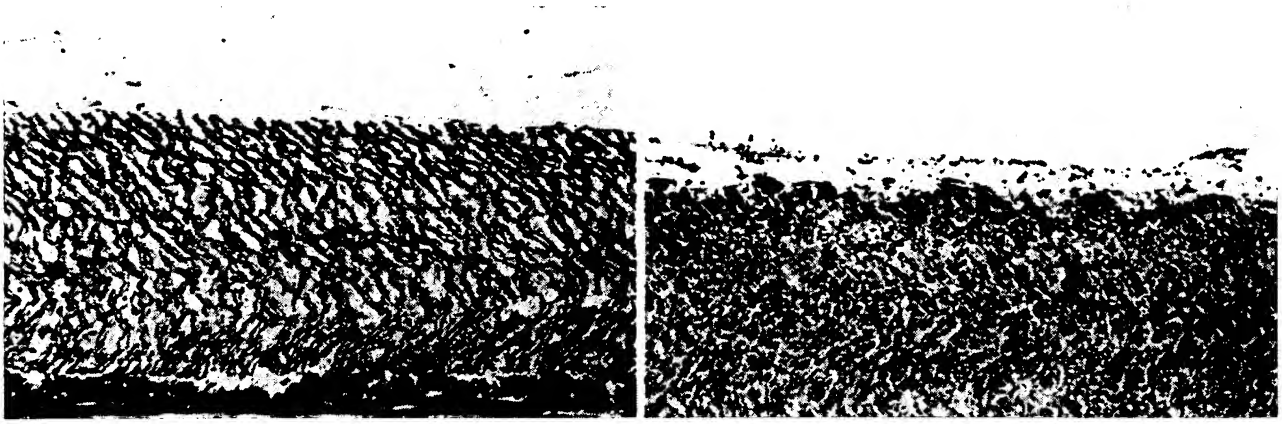

Fig. 1. Implantation of polytetrafluorethylene tubes with adsorbed AGE albumin incites mononuclear phagocyte migration. Polytetrafluorethylene tubes incubated with either native rat serum albumin (left) or AGE rat serum albumin (right) were implanted subcutaneously into the backs of rats. After four days the tubes were removed and subjected to staining with hematoxylin and eosin. Strikingly increased numbers of mononuclear inflammatory cells were found in the AGE-coated polytetrafluorethylene, compared with that observed with the native albumin-coated polytetrafluorethylene.

mobilized RAGE was inhibited in the presence of excess unlabeled sRAGE. Similarly, in cell culture models, excess sRAGE inhibited the binding of radiolabeled AGE albumin to bovine aortic endothelial cells (69), and of radiolabeled AGE β_2-microglobulin to human peripheral blood–derived mononuclear cells (49). Functionally, excess sRAGE inhibited AGE-mediated mononuclear cell chemotaxis (73), increased expression of vascular cell adhesion molecule-1 in endothelial cells (70), increased hyperpermeability of endothelial cell monolayers (91) and increased elaboration of tumor necrosis factor-α into mononuclear cell supernatants (49). These data suggested that sRAGE bound AGEs and prevented their interaction with, and activation of, cell surface RAGE. Consistent with these observations, in *in vivo* studies intravenous administration of AGE albumin into normal mice resulted in its rapid clearance from the circulation; this was in contrast to the markedly delayed clearance of radiolabeled albumin. The clearance of radiolabeled AGE albumin was significantly attenuated in the presence of sRAGE. However, consistent with its specificity for AGE-modified adducts, administration of sRAGE had no effect on the clearance of native albumin. In normal mice, administration of sRAGE inhibited AGE-mediated increased expression of IL-6 in liver tissue (69). In normal rats, administration of sRAGE suppressed vascular permeability mediated by infusion of syngeneic diabetic red blood cells (91). These data suggested that sRAGE could interfere with the ability of AGEs to interact with, and activate, cellular targets.

The critical test of this hypothesis, however, was whether sRAGE might exert protective effects in diabetic animals. To further explore this issue, we first characterized sRAGE. Rat and murine sRAGE were prepared in a baculovirus expression system, purified to homogeneity, rendered free of detectable levels of endotoxin, and prepared for testing in diabetic animals. ^{125}I-Rat sRAGE was prepared and infused into the jugular veins of either control non-diabetic rats or rats previously rendered diabetic with streptozotocin (91). In rats diabetic for 9–11 weeks, the $t_{1/2}$ for elimination of sRAGE was 21.7±0.43 hours, compared with 13.6±0.79 hours in non-diabetic, age-matched controls (91). In both cases, the major organ in which sRAGE appeared to deposit was the kidney. In both diabetic and non-diabetic rats, sRAGE in plasma appeared intact, by precipitability in trichloroacetic acid and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography.

An important complication of diabetes, even early in the disease, is the presence of vascular hyperpermeability. Clinically, the onset of microalbuminuria has been demonstrated epidemiologically to portend the development of significant vascular disease and its related increased morbidity and mortality within years of the first demonstration of abnormal urinary excretion of albumin (48, 88). In our first studies using sRAGE in diabetic rodents, we measured the tissue-blood isotope ratio. In these experiments, ^{125}I-albumin and ^{51}Cr-labeled red blood cells were used to test vascular permeability, which had been previously demonstrated to be elevated in spontaneously diabetic or streptozotocin-induced

diabetic rats (93). In this model after 9–11 weeks of diabetes, vascular hyperpermeability was increased in a range of organs, particularly the skin, intestine and kidney (91). At a lower dose of sRAGE (2.25 mg/kg; intravenously), hyperpermeability was blocked in diabetic intestine and skin, and largely blocked (approximately by 60%) in diabetic kidney. However, at the higher dose of sRAGE, hyperpermeability in diabetic kidney was reversed by approximately 90% (Fig. 2). These beneficial results occurred within 1 hour of administration of sRAGE. Consistent with an important role for AGE-RAGE-mediated enhanced cellular oxidant stress in the pathogenesis of diabetic hyperpermeability, infusion of the antioxidant probucol into diabetic rats significantly attenuated hyperpermeability in a number of organs (91). Similarly, in another model of permeability, administration of sRAGE in streptozotocin-induced diabetic rats blocked increased lung hydraulic conductivity (7). Together, these data provided our first experimental evidence that administration of sRAGE might affect established diabetic complications.

While these data suggested efficacy of sRAGE in acute complications of diabetes, such as hyperpermeability, further studies have suggested efficacy of sRAGE in chronic complications of diabetes. We have demonstrated that administration of murine sRAGE ameliorated impaired wound healing in insulin-resistant, hyperglycemic db+/db+ mice subjected to full-thickness excisional wounds (94). In addition, we recently reported a model of advanced atherosclerosis in diabetic mice deficient in apolipoprotein E (60). Treatment of these mice with murine sRAGE suppressed atherosclerosis in a dose-dependent manner. Examination of the vascular lesions at the aortic sinus demonstrated diminished numbers of macrophages and smooth muscle cells in diabetic mice treated with sRAGE, suggesting that blockade of RAGE strikingly suppressed cellular activation. Importantly, the beneficial effects of sRAGE occurred in a glycemia- and lipid-independent manner, thus highlighting a novel axis in the development of vascular lesion formation in the diabetic milieu. Indeed, in our studies to date, there has been no evidence of systemic toxicity, even in mice treated with daily injections of sRAGE for up to 6 months. These data strongly suggest that sRAGE might represent a template for the design of novel therapeutic agents for diabetic complications.

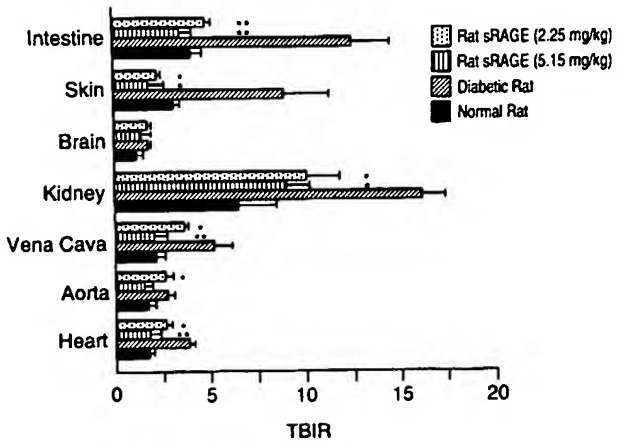

Fig. 2. Soluble RAGE blocks vascular hyperpermeability in diabetic rats. Normal rats or rats rendered diabetic with streptozotocin were killed after 9–11 weeks of diabetes and permeability was measured in a range of organs by tissue-blood isotope ratio (TBIR). As indicated, diabetic rats were treated with sRAGE at two different doses (2.25 and 5.15 mg/kg) in order to achieve expected sRAGE plasma concentrations of 10–20 or 40–60 μg/ml, respectively, prior to measurement of tissue-blood isotope ratio. Treatment of diabetic rats with soluble vascular cell adhesion molecule-1 had no effect (not shown).

Diabetes-associated periodontal disease: development of murine models to test the role of AGEs and RAGE

AGEs accumulate in human diabetic gingiva, similarly to trends observed in a range of other tissues (72). In our first studies, we harvested gingival tissue from patients undergoing surgery for moderate to advanced chronic adult periodontitis and analyzed the tissue extracts for AGE content. Significantly increased AGE levels were identified by enzyme-linked immunosorbent assay in the gingival extracts from diabetic patients, compared with non-diabetic controls (Fig. 3). Consistent with these findings, increased AGE-immunoreactive epitopes were identified by immunohistochemistry within the vasculature and connective tissue of human diabetic gingiva, compared with that observed in gingiva from non-diabetic patients (72). In adjacent sections, increased vascular staining for heme oxygenase-1, a marker of enhanced oxidant stress, was evident in diabetic gingival tissue when compared to tissue from non-diabetic periodontitis patients (72). These data, therefore, suggested that enhanced accumulation of AGEs and increased oxidant stress in human diabetic gingival tissue might play a role in the pathogenesis of diabetes-asssociated periodontitis.

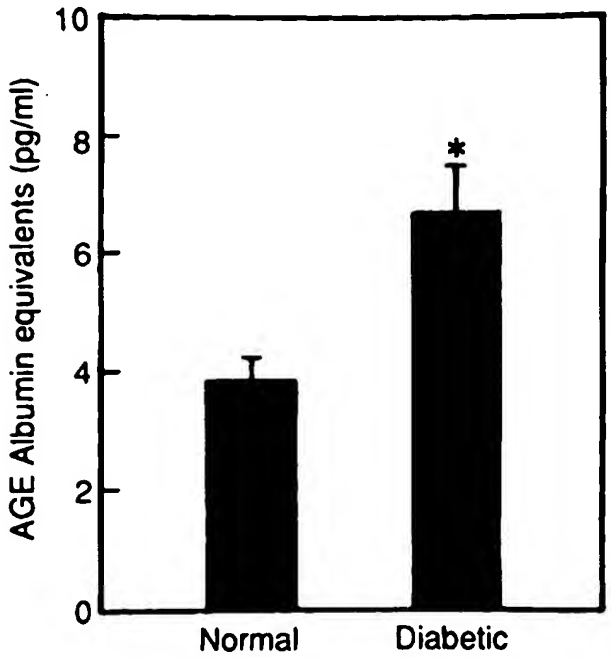

Fig. 3. AGE-immunoreactive material is increased in gingival tissue from human diabetic subjects. Gingival tissue was harvested from patients undergoing surgery for moderate to advanced chronic adult periodontitis and assayed by enzyme-linked immunosorbent assay using affinity-purified anti-AGE IgG. Five non-diabetic patients (mean age 32 years; range 21–40) and four diabetic patients (mean age 38 years; range 29–41) were included. The difference in the patients' ages was not statistically significant and no patient had renal failure. One of the diabetic patients had type 1 diabetes and the other three had type 2. AGE levels, expressed as AGE albumin equivalents, were 3.8 pg/ml versus 6.8 pg/ml, respectively. *$P<0.05$.

These considerations highlighted the importance of developing small animal models of diabetes-associated periodontitis in order to dissect the role of AGEs and RAGE. Most of the available models to study mechanisms and therapeutic strategies in periodontal disease had been in non-human primates, dogs and rats (13, 24, 31, 38, 86). The study of mice, though, presented a particularly attractive model system, since the wide availability of genetically modified mice renders this species an ideal opportunity to rigorously delineate the importance of specific molecular and host response factors in the pathogenesis of periodontitis.

Therefore, we rendered male C57BL/6J mice diabetic with streptozotocin, a model of relative insulin deficiency or type 1 diabetes (41). Control mice were treated with citrate buffer alone. One month after documentation of diabetes or non-diabetic control state, mice were inoculated with the human periodontal pathogen *Porphyromonas gingivalis*, strain 381, or treated with vehicle (phosphate-buffered saline). At 2 and 3 months after infection, increased alveolar bone loss and collagenolytic activity were demonstrated in *P. gingivalis*–infected, diabetic versus non-diabetic mice (Fig. 4, 5). Consistent with our hypotheses that enhanced formation and accumulation of AGEs, and expression of RAGE contributed, at least in part to these observations, increased AGEs and vascular and monocyte expression of RAGE were demonstrated in diabetic gingiva by immunohistochemistry (41).

Models of murine type 2 diabetes are also available, such as the highly insulin-resistant, hyperglycemic db+/db+ mouse. Indeed, pilot studies from our laboratory have revealed that after inoculation with *P. gingivalis*, alveolar bone loss is also increased in these mice, compared with their age-matched non-diabetic m+/db+ controls.

In-depth studies are in progress to determine the factors that increase susceptibility to alveolar bone loss in diabetes, the specific effects of diabetes on the host response that appear to favor sustained inflammation and destruction in the periodontium, as well as the effects of blockade of the RAGE, by administration of sRAGE in this murine model of diabetes-associated periodontitis.

Hypotheses

Diabetes is associated with a broad range of complications, and a number of underlying mechanisms likely account for these. The direct toxic effects of elevated blood glucose can have important pathological consequences. However, evidence is accumulating to implicate an important role for indirect effects of hyperglycemia, such as the formation of AGEs. The interaction of AGEs with RAGE appears not to mediate clearance or neutralization of these modified structures, but rather, chronic cellular perturbation and dysfunction. Indeed, despite accumulation of AGEs in diabetes, the expression of RAGE is in fact enhanced and not downregulated as might be expected. The molecular cues that regulate expression of RAGE in such settings may be explained, at least in part, by regulatory elements within its promoter linked to the inflammatory response, such as nuclear factor-κB, nuclear factor–interleukin 6 and γ-interferon response elements (44).

Our studies have indicated that, both *in vitro* and *in vivo*, the interaction of AGEs with cellular RAGE is inhibited in the presence of sRAGE or anti-RAGE IgG and, therefore, suggest an important role for this in-

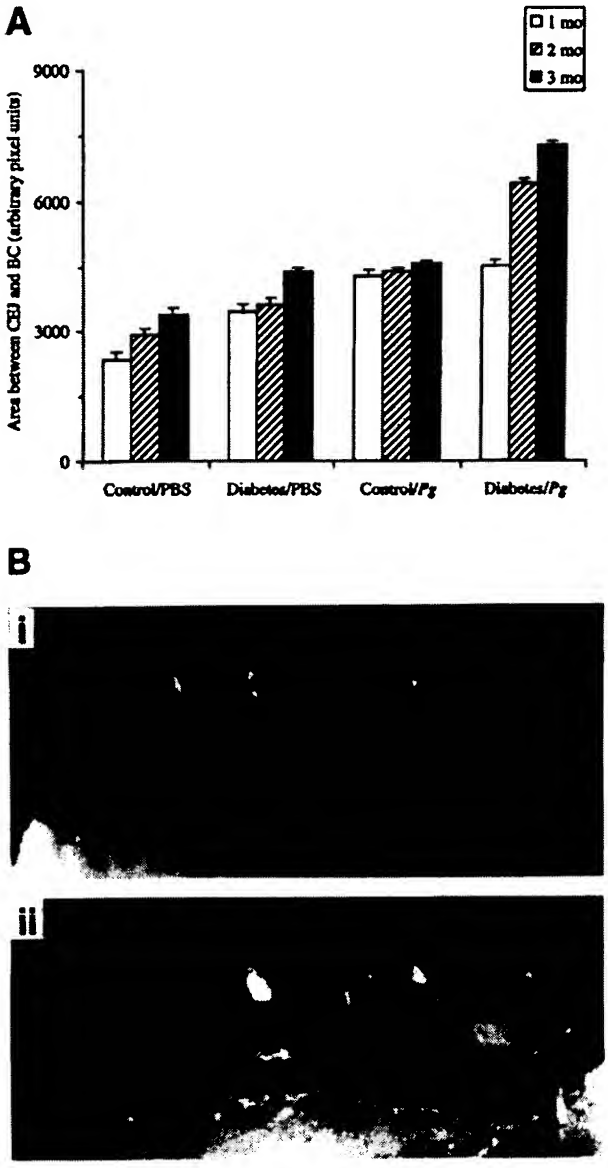

Fig. 4. Accelerated alveolar bone loss develops in diabetic mice infected with *P. gingivalis*. **A.** Morphometric analysis. Bone loss was defined as the total area between cementoenamel junction and alveolar bone crest for all six posterior mandibular teeth, and is reported in arbitrary pixel units. Based on standardization with known units of area, 12,210 pixels=1 mm^2. Groups are as follows: "control" indicates non-diabetic mice, "diabetes" indicates mice in whom diabetes was induced with streptozotocin, "PBS" indicates local treatment with vehicle (phosphate-buffered saline) and *Pg* indicates inoculation with *P. gingivalis*. Results for all four groups are shown at 1, 2 and 3 months after infection or vehicle treatment. Importantly, at 2 and 3 months after *P. gingivalis* infection, diabetic animals demonstrated a statistically significant increase in alveolar bone loss as compared with non-diabetic animals. **B.** Representative photographs of mandibles. The lingual surfaces of posterior teeth are shown in half mandibles obtained from a representative non-diabetic mouse treated with phosphate-buffered saline (I) and a diabetic mouse infected with *P. gingivalis* (II), both at 2 months. The shaded areas between the cementoenamel junction (CEJ) and alveolar bone crest (BC) were computer-analyzed and measured to evaluate differences in alveolar bone loss between groups.

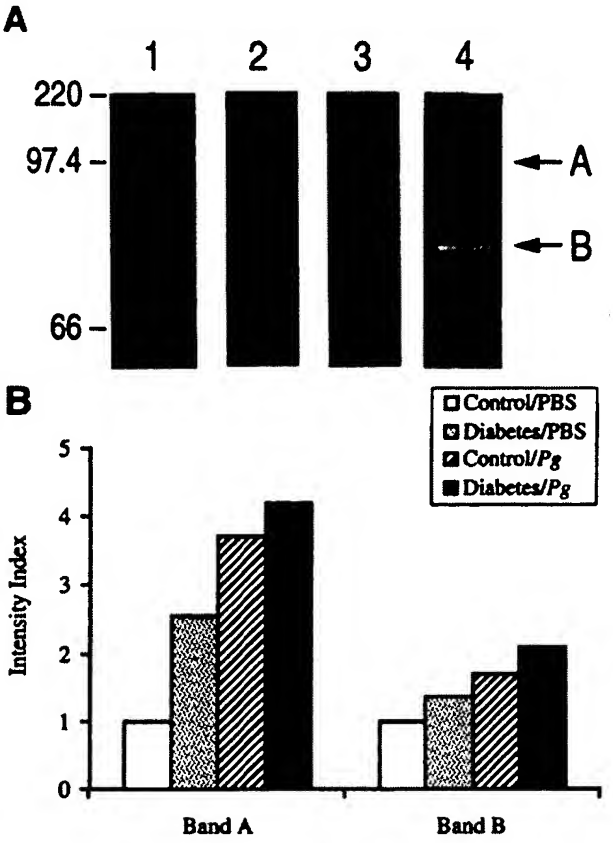

Fig. 5. Enhanced collagenolytic activity is observed in gingival extracts of diabetic mice. **A.** Zymographic analysis. Lane 1: non-diabetes/phosphate-buffered saline, lane 2: diabetes/phosphate-buffered saline, lane 3: non-diabetes/*P. gingivalis* and lane 4: diabetes/*P. gingivalis*. At 2 months after either infection with *P. gingivalis* or vehicle treatment with phosphate-buffered saline, gingival tissue extracts were prepared and subjected to chromatography onto gels containing gelatin. Two major bands of collagenolytic activity were observed, indicated as bands A and B. Approximate molecular weights were deduced from molecular weight standards as indicated. **B.** Densitometric analysis. Densitometric analysis of bands A and B was performed. Intensity of band A or B in non-diabetic mice treated with phosphate-buffered saline (PBS) was arbitrarily considered as "1" in each case. Analyses of bands in diabetes/phosphate-buffered saline, control/*P. gingivalis* (*Pg*) and diabetes/*P. gingivalis* was made relative to the intensity of bands A or B in control/phosphate-buffered saline mice. Increased collagenolytic activity was observed in the gingival extracts of the diabetic *P. gingivalis*–infected mice compared with that observed among the other groups. Indeed, the extend of collagenolytic activity paralleled that of alveolar bone loss among the groups of mice tested.

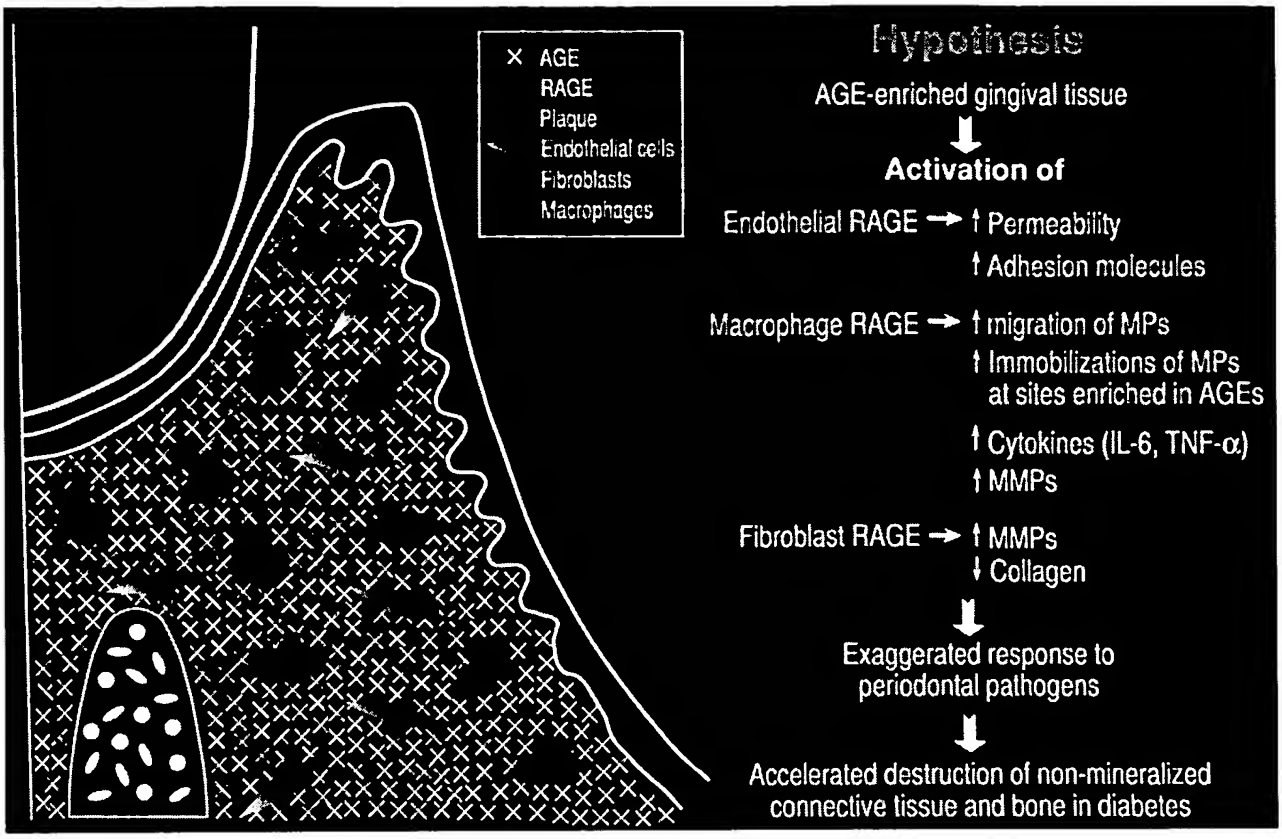

Fig. 6. Influence of AGEs and RAGE on diabetic periodontium. This figure summarizes our hypotheses regarding the potential role of enhanced AGE interaction with cellular RAGE in the pathogenesis of diabetes-associated periodontal disease. MPs: macrophages. IL-6: interleukin-6. TNF-α: tumor necrosis factor α. MMPs: matrix metalloproteinases.

teraction in the pathogenesis of vascular and inflammatory cell activation in diabetes. This ligand-receptor binding, over long periods of time, may lead to progressive vascular disease and irreversible tissue destruction.

As summarized in Fig. 6, we speculate that increased accumulation of AGEs and their interaction with RAGE in diabetic gingiva leads to vascular dysfunction with hyperpermeability, and loss of effective tissue integrity and barrier function. Increased tissue AGEs within the gingiva may serve as a locus for the attraction, immobilization and activation of mononuclear phagocytes, critical mediators in the generation of proinflammatory cytokines and matrix metalloproteinases (62). Further inflammatory cell influx and activation in the diabetic gingiva may ensue from vascular activation, with enhanced expression of adhesion molecules. Activated fibroblasts, in addition to potentially producing further tissue destructive mediators, may also demonstrate diminished reparative responses, with decreased collagen synthesis.

In conclusion, a "two-hit" model of cellular perturbation may be envisioned. When infection with periodontal pathogens occurs in an AGE-enriched environment, exaggerated and sustained inflammatory responses ensue in a RAGE-dependent manner. When further superimposed on a setting of impaired reparative responses, destructive periodontal disease results.

Future directions and challenges

An important challenge in this work is to identify the importance of RAGE in the enhanced periodontal inflammation and attachment loss that occurs in diabetic individuals.

Experiments in which sRAGE is administered to diabetic mice infected with a major periodontal pathogen are the first test for the hypothesis that limiting access of AGEs to cellular RAGE will prevent or delay cellular activation, which eventuates in complications such as accelerated periodontal disease. Furthermore, mice in whom the genetic expression of RAGE is modified are also being gener-

ated. For example, overexpression of full-length RAGE in macrophages and endothelial cells should be helpful. In addition, mice in whom the ability of AGE ligation of RAGE to activate RAGE-dependent cell-signaling pathways is abrogated (such as those in whom the cytosolic domain of the receptor has been deleted), are also being generated. Together with mice in whom the expression of RAGE has been deleted, such studies should resolve whether neutralization of RAGE is protective, especially in an AGE–enriched environment.

The results of these studies should determine whether the AGE-RAGE interaction provides a logical and feasible target for directed therapy of diabetic complications, including destructive periodontal disease. Soluble RAGE may then represent a prototypic structure for the design of agents to be used towards this end.

Acknowledgments

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